Summer 8-13-2013

HCMV Promotes Breast Cancer Metastasis: Impacts of CMVIL-10 in the Tumor Microenvironment

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HCMV PROMOTES BREAST CANCER METASTASIS: IMPACTS OF CMVIL-10 IN THE TUMOR MICROENVIRONMENT

By

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Thesis
Submitted in Partial Fulfillment of the Requirements
For the Degree of

Master's of Science
In
Biology

In the

College of Arts and Sciences
Department of Biology
University of San Francisco
San Francisco, California

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Abstract

Human cytomegalovirus (HCMV) is a highly species-specific, common human pathogen. While a large majority of people are infected with HCMV worldwide, infection is typically asymptomatic in immune competent individuals. To enhance virus persistence and avoid immune detection by the host, HCMV exploits the strategy of encoding homologs of cellular cytokines. One factor produced by HCMV-infected cells is the viral cytokine cmvIL-10, which acts through the cellular IL-10 receptor to induce downstream cell signaling. In instances where HCMV-infected cells infiltrate the tumor microenvironment, the presence of cmvIL-10 in may initiate events that promote a more invasive tumor phenotype. Cancer cells that express fewer adhesion molecules, which allow cells to break off from the primary tumor, are more likely to metastasize. In addition, matrix metalloproteinases (MMPs) degrade the extracellular matrix, an effect which promotes cancer cell dissemination by permitting tumor cells access to the vascular and lymphatic systems. We found that MCF-7 human breast cancer cells express the cellular IL-10 receptor and that treatment with cmvIL-10 results in changes in gene expression and cellular activity related to cancer progression. We demonstrate that cmvIL-10 alters expression of cell adhesion molecules and increases MMP gene expression. Breast cancer cells also exhibit more robust migration in the presence of cmvIL-10. CmvIL-10 may cooperate with other factors secreted by HCMV infected cells, and the secretome of HCMV infected fibroblasts are characterized. The results indicate that breast cancer cells exposed to cmvIL-10 undergo changes that could lead to the formation of more invasive tumors. This observation suggests that HCMV-positive cancer patients may have the potential to develop more metastases and could benefit from the inclusion of anti-viral therapeutics in their treatment regimen.

Table of Contents

Page Number

List of Figures

Page Number

List of Tables

List of Abbreviations

α gene products	Immediate early gene products
ATCC	American Type Culture Collection
β gene products	Delayed early gene products
cDNA	Complementary DNA
CmvIL-10	HCMV interleukin-10
CPE	Cytopathic effects
DMEM	Dulbecco's Modification of Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle Medium Nutrient Mixture F12
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptors
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
γ gene products	Late gene products
gB	glycoprotein B
gH	glycoprotein H
gL	glycoprotein L
HCMV	Human cytomegalovirus
HER2	Human epidermal growth factor receptor-2
ie1/ie2	Major immediate early locus
IF	Immunofluorescence
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL-10R	Cellular IL-10 receptor
LAcmvIL-10	Latent-associated cmvIL-10
MHC	Major histocompatibility
MIEP	Major immediate early promoter/enhancer
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MT-MMPs	Membrane-type matrix metalloproteinases
PBS	Phosphate-buffered saline
PDGFRα	Platelet-derived growth factor receptor α
qRT-PCR	Quantitative real-time polymerase chain reactions
RNA	Ribonucleic acid
STAT3	Signal transducer and activator of transcription-3
TGF-β	Transforming growth factor-β
TIMPs	Tissue inhibitor of metalloproteinases
U_L	Unique long
U_S	Unique short

Introduction

Human cytomegalovirus (HCMV) is a highly species-specific, common human pathogen infecting people worldwide. Seroprevalence of infection in the United States ranges from 50-90% for middle aged adults (65, 78) and correlates directly to exchange of bodily fluids (18). Factors elevating HCMV incidence between populations include poor socioeconomic conditions and high-risk sexual practices (14, 18, 49). Seroprevalence may be greater than 95% in developing countries (65).

While a large majority of people are eventually infected with HCMV, infection is typically asymptomatic in healthy individuals. However, in immunocompromised hosts such as transplant recipients and HIV patients, HCMV can produce serious illness or fatal conditions (14, 71). Additionally, the developing fetus is threatened when a pregnant woman experiences primary exposure to HCMV. Congenital cytomegalovirus infection is the leading infectious cause of mental retardation and sensorineural hearing loss in the United States, affecting about 8,000 American children per year (8). Furthermore, while infection in the immunocompetent host is generally asymptomatic, HCMV actively modulates multiple cell regulatory and signaling pathways in healthy HCMV seropositive humans (9). Such manipulations of cellular pathways result in extensive micro-effects in the host organism, such as modification of the cell cycle and regulation of the immune system. These pervasive health concerns illustrate compelling reasons for continued investigation of the biology and immunology of HCMV.

HCMV belongs to the β subgroup of the Herpesviridae family and, like other herpesviruses, establishes lifelong latency in the host. HCMV contains a large double-stranded, linear DNA genome of 235kb in length. HCMV DNA is composed of two unique components denoted Unique Long (U_L) and Unique Short (U_S), which are each flanked by direct and inverted repeat elements (Figure 1) (35). The chromosome is able to isomerize at the junction of the two

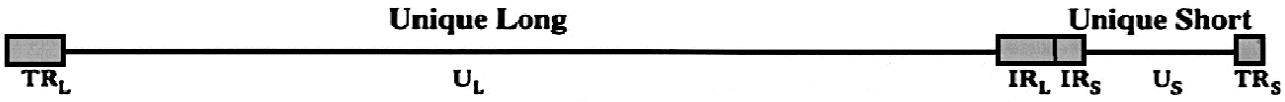

Figure 1. A schematic map of the HCMV genome. The HCMV genome is organized as two unique sequences (U_L) and (U_S) flanked by two sets of repeat elements, TR and IR, long($_L$) and short($_S$). Figure adapted from Kotenko et. al., 2000 (35).

segments to create four possible different genomic isomers (46), each of which encode for 165 HCMV genes (17). The DNA core is enclosed in a 100 nm diameter icosahedral capsid and surrounded by a tegument of phosophoproteins. These components are enveloped by a lipid bilayer carrying an abundance of viral glycoproteins. The entire virus particle ranges in size from 150-200 nm (46).

HCMV infects many human cell types *in vivo*, including epithelial cells, fibroblasts, monocytes, macrophages, and lymphocytes. In cell culture, however, HCMV exhibits a highly restrictive host range, preferring primary differentiated skin or lung fibroblasts (46). The cellular determinants of HCMV tropism and attachment are numerous, and many have still not been completely identified (15, 45). Basically, HCMV binds to cell surfaces by the interaction of virion envelope proteins, often glycoprotein B (gB), with heparan sulfate proteoglycans (16), which concentrates virions on cell surfaces to promote other interactions with cellular receptors (5).

HCMV entry into the cell is not straightforward or uniformly agreed upon by researchers. The cellular receptors utilized for viral entry, particularly Platelet Devived Growth Factor Receptor α (PDGFRα), are debated (61, 72). It is widely hypothesized that the envelope glycoproteins which HCMV employs to gain entry vary according to cell type being infected (28). Entry of HCMV into all human cell types involves at least viral envelope glycoprotein H (gH) and glycoprotein L (gL). For infection of epithelial and endothelial cells, leukocytes, and monocytes, HCMV assembles a glycoprotein complex composed of gH/gL and three other virally encoded proteins: UL128, UL130, and UL131 (72). The virion is then internalized by endocytosis followed by fusion with endosomal membranes (56). In contrast, this five glycoprotein complex is not required for viral entry into human fibroblasts. Here the viral

envelope, assisted by gH/gL and likely driven by the interaction of gB and integrins (26), fuses directly with the cellular plasma membrane and the viral capsid is deposited into the cytoplasm. These two different pathways of entry require distinct combinations of HCMV envelope glycoproteins.

The genome-carrying capsid is not the only organic material deposited into the cell upon virus entry. The tegument layer is an area between the lipid envelope and the capsid in HCMV virions that harbors over 100 distinct virally-coded and cellular proteins (33). These tegument proteins are dispensed into the cellular space and serve, in part, to mediate the delivery of the capsid along microtubules to the cell nucleus (33). Additional tegument proteins migrate independently to the nucleus, while others remain in the cytoplasm. Further tegument material includes mRNA ready for immediate translation by cellular ribosomes (6). In this critical stage of infection when the virus will commit to active replication or latency, the proteins and mRNA from the tegument layer begin to establish active regulatory systems that determine the activity of the infected cell (25). In this way, HCMV modulates the host cellular environment before the viral genome even arrives at the nucleus for transcription.

HCMV genome replication is slow compared to other herpesviruses, confined by early and late stage gene expression. Upon entry into the host nucleus, the viral tegument protein pp71 commits the virus to the lytic cycle by performing as a transactivator in the nucleus, promoting activation of the strong Major Immediate Early Promoter/enhancer (MIEP) (33). MIEP is upstream of the major IE locus (ie1/ie2) containing genes encoding α (Immediate early) gene products. MIEP is a very strong enhancer region employing a plethora of binding sites for cellular transcription factors and viral proteins (46). Host cell RNA polymerase II recognizes viral gene promoters and transcribes the nuclear phosphoprotein gene products IE1 and IE2.

These proteins are involved in both positive and negative regulation of viral gene expression. HCMV IE1 autostimulates its own synthesis through activation of MIEP and modulation of the cellular environment, further encouraging MIEP (15). HCMV IE2 shuts off ie1/ie2 and transitions viral expression from α to β (delayed early) gene expression. HCMV DNA polymerase, as well as origin binding proteins and other viral replication proteins, are translated by cellular ribosomes and transported to the nucleus during early gene expression as cellular metabolism is stimulated (46). Viral DNA replication begins at the oriLyt replication origin. γ (Late) gene expression ensues 24-36 hours after infection, encoding for structural proteins and proteins enabling progeny virus assembly (46). Maximum HCMV replication occurs 72-96 hours post-infection, and may persist for one week (25).

Overall, lytic cycle HCMV infection (Figure 2) results in viral gene expression and replication and is contingent upon cell vitality and activity. Unlike other herpesviruses, HCMV DNA proliferation is dependent on cellular DNA replication, so virus replication is more rapid in active cells than resting cells. Acute HCMV infection stimulates cellular nucleic acid processing and synthesis of gene products in order to create an optimum, active host for virus proliferation. The ability of HCMV to stimulate cellular metabolism enough to permit virus replication, yet avoid immune system clearance in an organism under strict surveillance is impressive.

While stimulating cellular metabolism, the virus needs to avoid recognition in order to persist in its host. This challenging dichotomy demonstrates a substantial evolutionary pressure to develop strategies to avoid detection and elimination by the human immune system. First, HCMV prefers to replicate in tissues with less stringent immune surveillance, such as the epithelial cells of the salivary glands, which do not express a sufficient number of major histocompatibility complex (MHC) class I molecules to coordinate a complete immune response

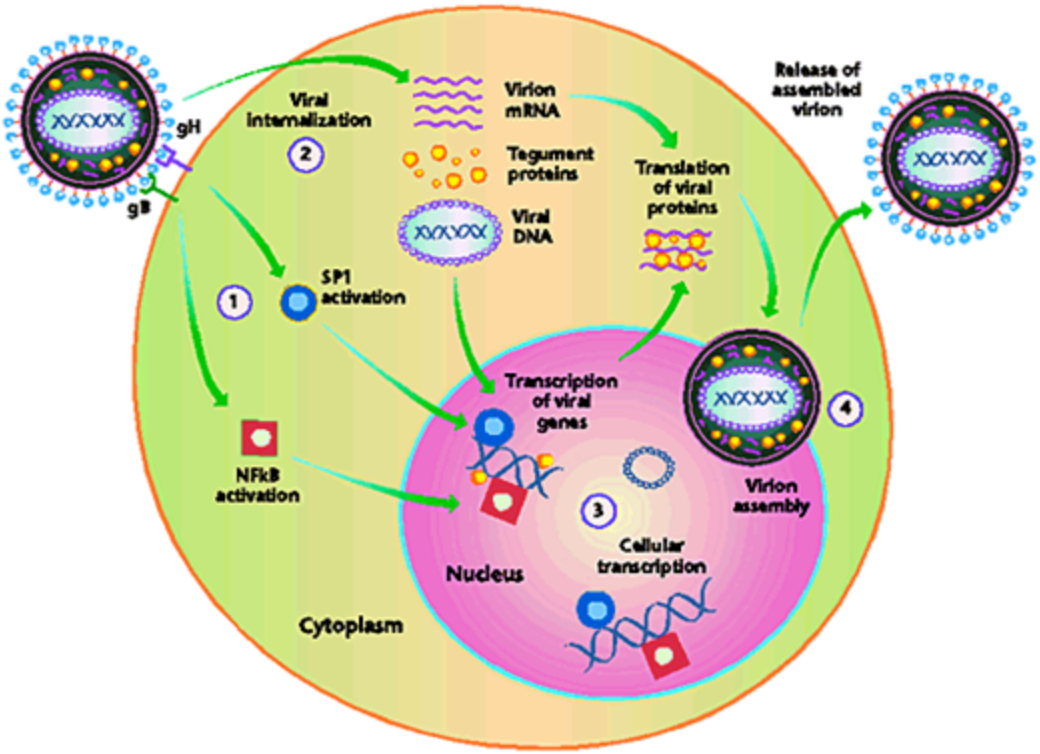

Figure 2. A schematic map of the HCMV lytic cycle. 1. Cellular transcriptional factors are activated. 2. Viral mRNA is translated. Viral DNA is transported to the nucleus. 3. Activated transcriptional factors encourage viral and cellular DNA expression in the nucleus. Viral DNA is replicated. 4. Viral DNA, viral and cellular proteins and virion transcripts are packaged. Infectious virus is released. Figure adapted from Huang, et al., 2000 (25).

(24). For this reason HCMV was once known as "salivary gland virus," and was first isolated from greatly enlarged cells, or cytomegalic inclusion bodies, of the salivary glands (63). Newly assembled viruses are seamlessly transmitted to new hosts in saliva, permitting dissemination. HCMV does not, however, merely hide in salivary glands to avoid immune detection by the host, but actively orchestrates its evasion from immune clearance. As mentioned, HCMV is capable of infecting a broad array of cell types and, like other herpesviruses, HCMV establishes latent infection at some point following primary productive infection (59).

Latent infection is the absence of detectable virus despite the presence of viral DNA; HCMV commences a life-long co-existence with the host. Latent HCMV is believed to reside primarily in myeloid progenitor cells in the bone marrow (58), but not all sites for latency are documented. Latency involves strategies to allow viral persistence through tactics of evasion from immune system clearance, despite an enormous anti-HCMV response by $CD4^+$ and $CD8^+$ T cells (40).

Cells of the human immune system rely on a system of secreted proteins including cytokines, chemokines, and their receptors to interact with each other and coordinate an effective immune response. Cytokines are proteins secreted by cells to convey information to other cells. Cells that have the appropriate receptor for the cytokine are capable of receiving the message and transmitting the signal inside the cell via intramolecular pathways (63). Chemokines, or chemotactic cytokines, are a subtype of cytokine that attract immune cells by inducing migration to sites of infection or damage. In infected cells, HCMV can produce over 250 proteins, but only about 50-60 are essential for viral replication (60). Thus, the vast majority of viral proteins enable the virus to persist with the host. HCMV has hijacked the cellular language of cytokines and chemokines by developing numerous homologs of cellular cytokines and cytokine receptors as a strategy to limit immune control, a phenomenon known as molecular mimicry. The latent

infection launched by HCMV involves the production and secretion of these immunomodulatory homologs to restrict the cell cycle, evade apoptosis and diminish immune recognition and response (63). These proteins act through highly sophisticated mechanisms to control, silence, and counteract normal immune mechanisms in order to facilitate viral production and to avoid detection and elimination of the virus.

One factor expressed by HCMV to manipulate the host environment is HCMV interleukin-10 (cmvIL-10). CmvIL-10 is a homolog of human IL-10, a cellular cytokine primarily involved in immune suppression. Human IL-10 has effects on many cell types, but predominantly regulates the immune response by targeting monocytes to inhibit production of cytokines, including IL-1α, IL-1β, IL-6, and TNFα (63). A reduction of these cytokines limits the inflammatory response, counteracting activity mediated by interferon gamma (IFNγ). Additionally, human IL-10 down-regulates MHC class II and co-stimulatory molecules (19, 64) to decrease T-cell effector activity. The HCMV mimic of this cytokine, cmvIL-10, retains these same powerful abilities.

CmvIL-10 is encoded by the viral gene UL111A (Figure 3). During productive infection, the 175 amino acid protein is produced from splicing of the three exons and two introns of gene UL111A with late kinetics (35, 37). CmvIL-10 has 27% amino acid identity to human IL-10 yet binds to the cellular IL-10 receptor (IL-10R) with greater affinity than human IL-10 itself (32). Alternative splicing of the UL111A gene produces a smaller 139 amino acid protein denoted LAcmvIL-10 that is expressed with early kinetics in productive infection and continues to be expressed during latent infection (30, 31). For the purpose of this investigation, discussions of cmvIL-10 will focus on the larger, full-size version of the two transcripts. CmvIL-10 binds to cellular receptor IL-10R, activating the JAK/STAT3 pathway (Figure 4) (62). Initially, cmvIL-10 interacts with the extracellular domain of ligand binding subunit IL-10Rα, which is supported

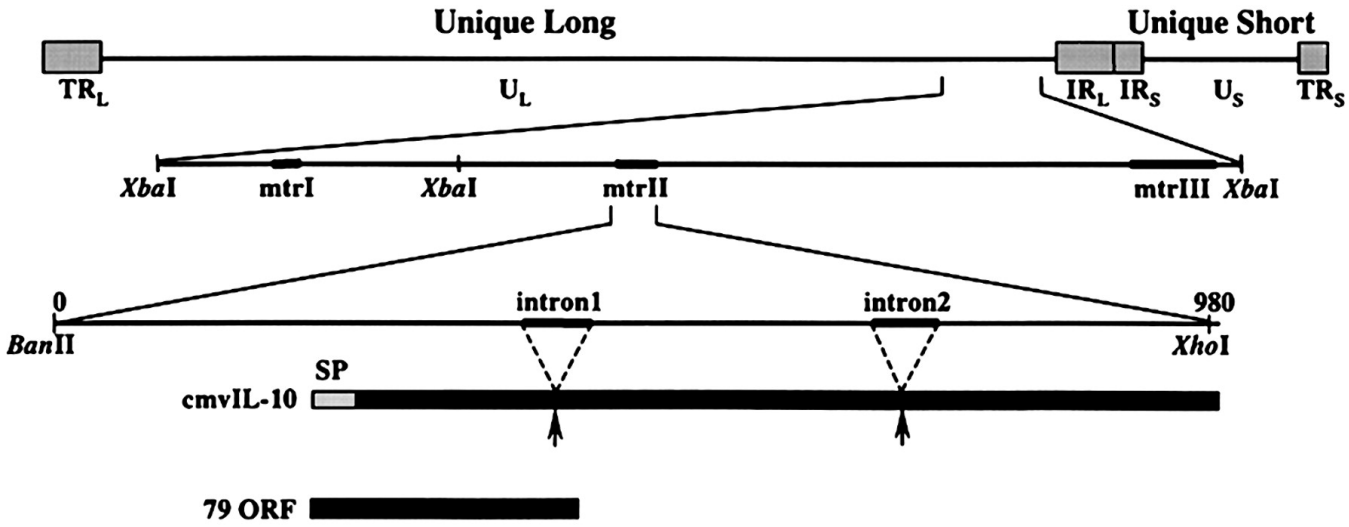

Figure 3. A schematic map of the gene encoding for cmvIL-10. The 175 amino acid cmvIL-10 protein is encoded by three exons from the viral gene UL111A (ORF 79). Figure from Kotenko et. al., 2000 (35).

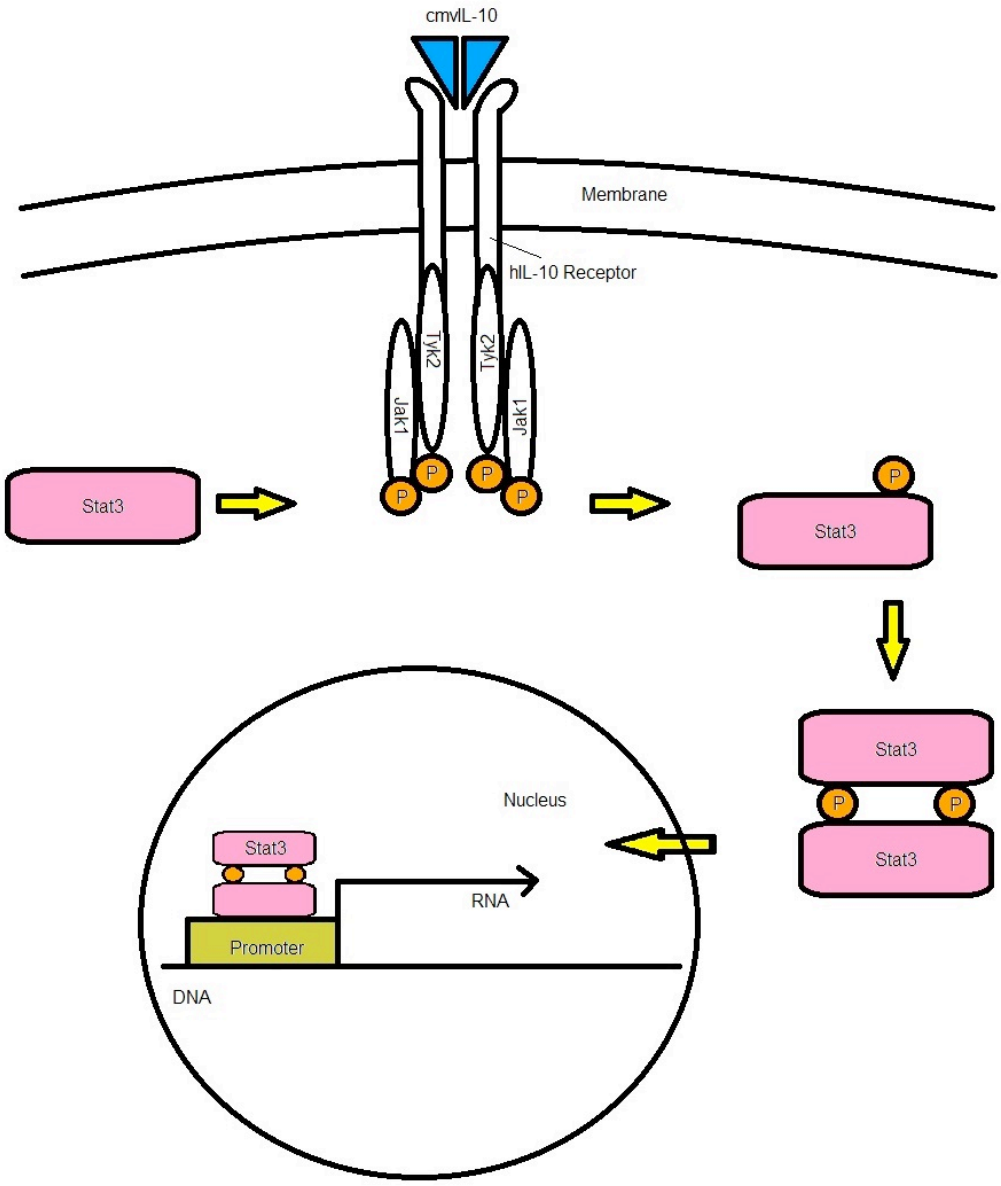

Figure 4. A schematic illustration of Stat3 activation by cmvIL-10. The binding of cmvIL-10 to the receptor results in phosphorylation of Jak1. Stat3 is recruited to the phosphorylated receptor and also phosphorylated. Stat3 dimerizes and migrates into the nucleus, where it binds to the promoter of target genes, stimulating expression.

by signaling subunit IL-10Rβ. The interaction engages the tyrosine kinases Jak1 and Tyk2 that are constitutively associated with the intracellular portions of the receptor. Phosphorylated Jak1 and Tyk2 cross-phosphorylate the intracellular chain of IL-10Rα. STAT3 (signal transducer and activator of transcription-3) docks to these phosphorylated tyrosine residues, binds via its SH2 domain, and is phosphorylated. STAT3 homodimerizes, dissociates from the receptor, and translocates to the nucleus to bind to promoters and induce transcription (20). The resulting effects include inhibition of cytokine synthesis and downregulation of MHC class II molecules, resulting in a reduction in proliferation and differentiation of peripheral blood mononuclear cells of the immune system (31, 59, 64). In this way, cmvIL-10 promotes transcription of human IL-10-associated genes via STAT3 activation.

When cmvIL-10 is secreted from infected cells, the resulting suppressive effects are capable of affecting neighboring uninfected cells in a paracrine fashion (47, 64). HCMV can employ the properties of cmvIL-10 to manipulate the host cell and surrounding uninfected tissue to silence signaling mechanisms, resulting in a failure to attract local immune cells to the site of infection. The dampening of host defense mechanisms through the expression of cmvIL-10 establishes a more preferable environment for the virus. However, the potent immunosuppressive activities of cmvIL-10 may have vast implications for the immunocompetent host in circumstances where cancerous cells are in the vicinity of HCMV infected cells. Specifically, research is emerging to suggest that the immunomodulatory proteins encoded by HCMV may also contribute to the promotion of common inflammation-related diseases, such as cancer (60). The term oncomodulation indicates that while HCMV is not regarded as an oncogenic virus, HCMV infection may promote malignancy of tumor cells. Current research suggests there is a connection between HCMV and cancer progression (11-13, 40-43) (Figure 5).

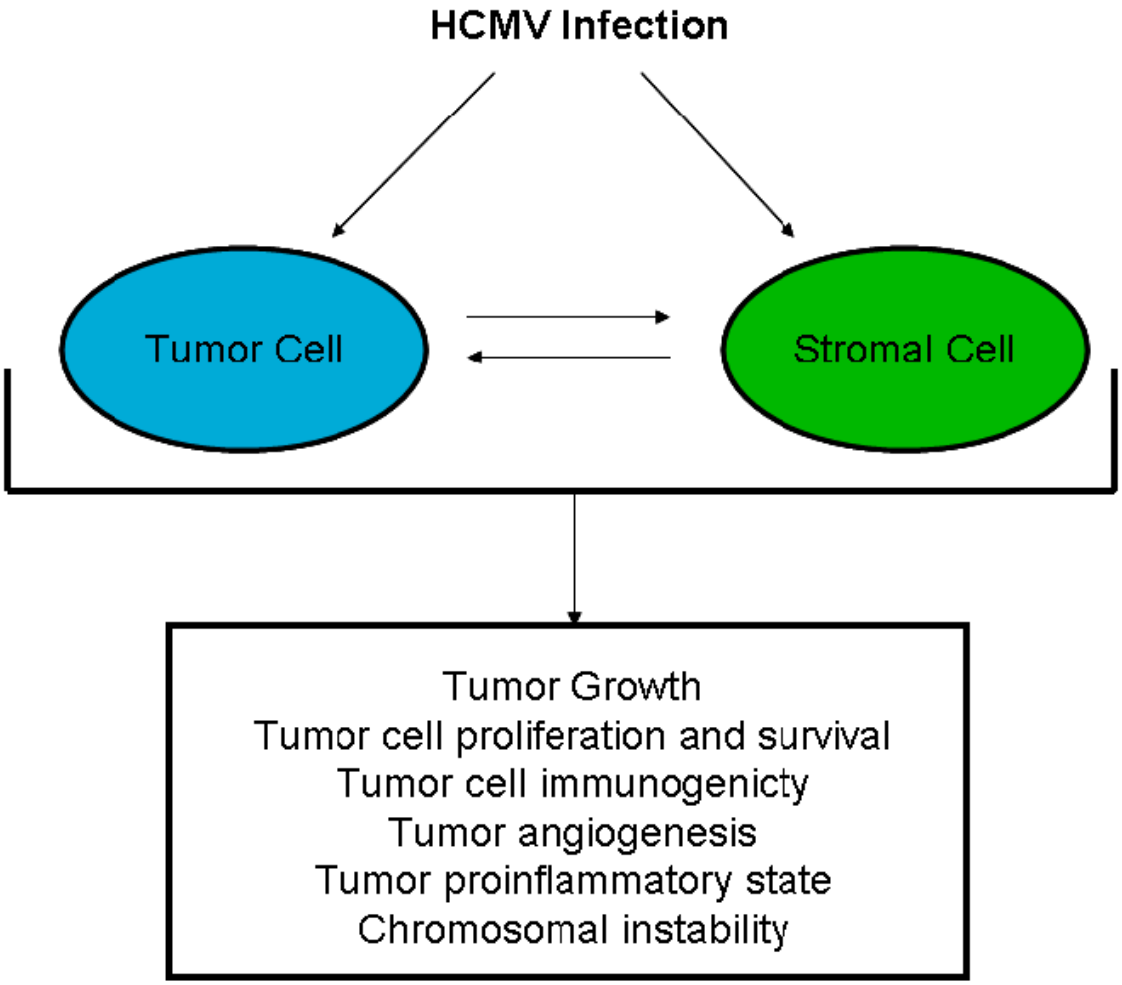

Figure 5. HCMV may infect tumor and stromal cells, resulting in increased tumor malignancy. The interaction between HCMV-infected or non-infected cell types may be crucial for oncomodulatory effects. Figure adapted from Michaelis et. al., 2011 (41).

Furthermore, cancer involves a genetic mutation in a cell that leads to uncontrolled proliferation, which could be exacerbated by the virally-altered immunoenvironment (10). Expression of HCMV gene products in or around an established tumor may accelerate progression of the tumor by promoting various signaling pathways critical to tumor growth such as mitogenesis, apoptosis, inflammation, angiogenesis, invasion and migration (11, 42). The present research investigates the effects of viral cytokine cmvIL-10 on breast cancer cell metastasis.

Metastatic breast cancer presents a colossal issue for women's health. In the United States, there are over 200,000 new diagnoses of invasive breast cancer annually. Nearly 40,000 American women died of breast cancer in 2012 (1). Diverse genetic mutations are responsible for the development of breast tumors, and there currently exist four molecular subtypes of breast cancer (70). Subtype Luminal A consists of tumors expressing estrogen receptors (ER) on the surface but not human epidermal growth factor receptor-2 (HER2). Subtype Luminal B includes tumors with estrogen receptors and HER2 on the cell surface. Triple-Negative Subtype tumors have little or no estrogen receptors or HER2. HER2 Subtype tumors have little or no estrogen receptors but are HER2 positive (70). Table 1 provides the four breast cancer molecular subtypes and corresponding prevalence.

Metastatic breast cancer is an advanced stage of breast cancer where a cancerous cell or group of cells has uprooted from the primary tumor in the breast and established a secondary tumor in another organ or part of the body (Figure 6). Between one-half and two-thirds of American women diagnosed with Stage II and III breast cancer will develop at least one secondary tumor within 5 years of diagnosis (39). Despite persistent screening efforts and preventative measures, one in eight women will develop metastatic breast cancer in their lifetime

Table 1. Molecular subtypes of breast cancer tumors.

Subtype	These tumors tend to be	Prevalence (approximate)
Luminal A	ER^+ and/or PR^+, $HER2^-$, low Ki67	40%
Luminal B	ER^+ and/or PR^+, $HER2^+$ (or $HER2^-$ with high Ki67)	20%
Triple negative/basal-like	ER^-, PR^-, $HER2^-$, cytokeratin $5/6^+$ and/or $HER1^+$	15-25%
HER2 Subtype	ER^-, PR^-, $HER2^+$	10-15%

*Adapted from Susan G. Komen for the Cure (68).

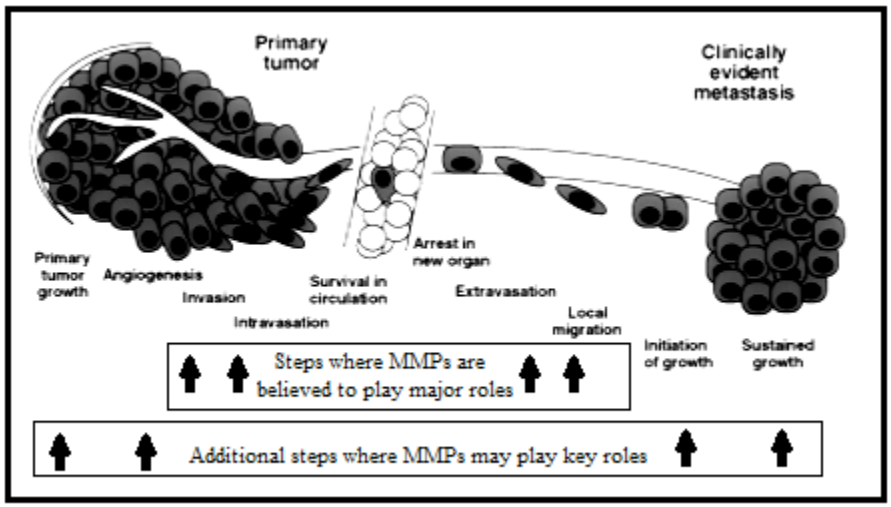

Figure 6. Metalloproteinases play a significant role in the metastatic process. MMPs are associated with steps in the metastatic cascade involving matrix degradation, invasion and local migration. Figure adapted from Chambers et. al., 1997 (7).

and one in 36 women will die from the disease (1). The metastatic process begins with invasion, in which the cancer cell breaks through the basal lamina of the primary tissue. The cell enters the lymph or adheres to a capillary wall. Intravasation involves the cell invading the capillary or being deposited into blood from the lymph and joining the circulatory system. The cancer cell is violently transported to another part of the body and a vast majority of cancer cells do not survive this journey. Extravasation entails the cancer cell escaping from the blood vessel in the new region. The cancer cell migrates locally and begins to establish residence by adapting and transitioning to conform to the surrounding tissue. The cancer cell commences to proliferate and develops into a secondary tumor (75). Breast cancer tumors metastasize most frequently to bone, lungs, lymph nodes, liver, and brain (50).

Invasion is promoted by the presence of matrix metalloproteinases (MMPs), a family of proteolytic enzymes that degrade the extracellular matrix (ECM) (73). MMPs permit malignant tumor cells access to the vascular and lymphatic systems, leading to cancer cell dissemination (Figure 6) (7). Elevated levels of MMP-13 have been found to correlate with poor prognoses in breast cancer patients (77). In addition, evidence implicates the role of MMP-2 and MMP-9 in breast cancer genesis and progression (21, 66). MMPs are inhibited by tissue inhibitor of metalloproteinases (TIMPs), wedge-like proteins with a continuous ridge that slots into the active site of the MMPs to prevent catalytic activity (48). Furthermore, transforming growth factor-β1 (TGF-β1) is a multifuctional cytokine that plays a critical role in cell proliferation, migration, and synthesis of ECM. Reported mechanisms of TGF-β1 activation include cleavage by metalloproteinases (69). Metastatic cancer progression, driven by MMPs, is a serious threat to the treatment and survival of cancer patients (51).

Considering the prevalent cmvIL-10 activity in modulation of the host cell environment in HCMV-positive individuals, the possibility of cmvIL-10 contributing to cancer cell metastasis is reasonable and worthy of investigation. Here we show that cmvIL-10 treated breast cancer cells demonstrate an altered gene expression profile involving decreased cell-to-cell adhesion molecules and increased MMP-7, MMP-9 and MMP-10 levels. Furthermore, cmvIL-10 increases MCF7 cell migration, compared to untreated breast cancer cells. These results demonstrate an oncomodulatory effect of cmvIL-10 that may promote breast cancer metastasis. Thus, HCMV and the cytokine cmvIL-10 offer viable therapeutic targets for the prevention of breast cancer progression that could contribute to the decline of breast cancer mortality.

Materials and Methods

Cells. MCF-7 (American Type Culture Collection, ATCC, Manassas, VA), an estrogen receptor-positive invasive ductal carcinoma breast cell line, was grown at 37°C in a humidified 5% carbon dioxide incubator in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 (DMEM/F12, GIBCO, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO). HFF-1 (ATCC), normal human foreskin fibroblasts, were maintained at 37°C in a humidified 5% carbon dioxide incubator and grown in Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech, Manassas, VA) supplemented with 15% FBS. Cells were subcultured twice weekly by treatment with trypsin (Mediatech, Manassas, VA).

Antibodies. The antibodies used in flow cytometry analyses were goat anti-IL-10Rα (R&D Systems, Minneapolis, MN, FAB6280P) and mouse anti-IL10Rβ (R&D Systems, FAB874P). The antibodies used as isotypes for comparison were, respectively, goat IgG (R&D Systems, IC108P) and mouse IgG_1 (R&D Systems, 554680). For immunofluorescence (IF) staining of MCF-7 cells, the antibodies used were rabbit anti-IL-10Rα (Santa Cruz Biotechnology, Santa Cruz, CA, sc-984, 1:100), followed by goat anti-rabbit-TRITC (Southern Biotech, Birmingham, AL, #4010-03, 1:150). The IF antibodies were diluted with antibody dilution buffer (1% BSA, 0.5% Tween-20, 1X PBS) by the dilution factor indicated by the manufacturer. HFF-1 cells were analyzed for HCMV infection by immunofluorescence staining using mouse anti-US27 and anti-US27 rabbit antiserum kindly provided by Dr. Barry Margulies (Towson University, MD, 1:100), goat anti-cmvUS28 (Santa Cruz Biotechnology, sc-28042, 1:50), or mouse anti-cmvIE1 (Millipore, MAB810, 1:100). These anti-virus antibodies were followed by the secondary antibodies goat anti-mouse-FITC (Santa Cruz Biotechnology, sc-2010, 1:100), goat anti-rabbit-

FITC (Santa Cruz Biotechnology, sc-2012, 1:100), donkey anti-goat-FITC (Santa Cruz Biotechnology, sc-2024, 1:100) and goat anti-rabbit-TRITC (Southern Biotech, #4010-03, 1:150) in antibody dilution buffer.

Virus Infection and Propagation. A 95% confluent monolayer of HFF-1 cells in a T75 flask was inoculated with HCMV strain AD-169 (ATCC) according to the suppler's instructions. The T75 flask was observed until >90% cytopathic effects (CPE) were obtained, requiring approximately 15 days. Viral propagation was originally maintained in a cell-associated manner, by harvesting infected HFF-1 cells with trypsin and dispensing $2x10^5$ cells onto a new 95% confluent monolayer of uninfected HFF-1 cells in a T75 flask. Viral stocks were obtained from the remaining infected HFF-1 cells by pelleting and resuspending the cells in 1 mL PBS. Infected cells were disrupted by sonification with a Branson Sonifier 250 (Cycle Duty 2, Output 2) for 30 seconds. The clarified sonicate was frozen at -80°C for future use. After optimizing the viral propagation procedure, viral propagation was achieved by inoculating a T75 flask containing a 95% confluent monolayer of uninfected HFF-1 cells with 1mL supernatant from a T75 flask of HFF-1 cells infected with HCMV at >90% CPE. This optimized procedure was repeated once weekly when >90% CPE was observed.

Flow Cytometry. MCF7 cells were grown to 80% confluency and then serum starved for 24 hours prior to harvesting. Cells were scraped into serum-free media, pelleted and resuspended in fluorescence activated cell sorter (FACS) buffer (PBS pH 7.4, 0.5% BSA, 0.02% sodium azide). Samples of $1x10^5$ cells each were stained with 5 µL phycoerythrin-conjugated polyclonal anti-human IL-10R antibodies in a total volume of 100 µL FACS buffer on ice in the dark for 60 minutes. The cells were washed three times and fixed in 100 µL cold FACS buffer plus 100 µL

PBS containing 2% paraformaldehyde. Each sample was analyzed using a Becton-Dickinson FACSCalibur Flow Cytometer and Cell Quest Pro software.

Immunofluorescence. MCF-7 cells were seeded on FBS-treated glass coverslips in 6-well plates at $2x10^5$ cells per well. The cells were allowed to adhere and grow for 48 hours to achieve 70-80% confluency. The media was removed and replaced with serum free media overnight. The cells were washed with PBS and fixed in 4% paraformaldehyde, then permeabilized with 0.2% Triton-X-100 in phosphate-buffered saline (PBS) for 15 minutes, followed by an ice cold acetone:methanol solution for 30 minutes at room temperature. MCF-7 cells were blocked with 10% FBS in PBS at 37°C for 1 hour, then stained with primary antibody for 1 hour, followed by secondary antibody for 30 minutes each at 37°C in the dark. Where indicated, HFF-1 cells were seeded on FBS-treated glass coverslips in 6-well plates at $7x10^4$ cells per well. The cells were allowed to adhere and grow for 96 hours to achieve >80% confluency, then infected using $5x10^4$ HCMV-infected cells. At 72 hours post-infection the cells were fixed, permeabilized and blocked as described above. HFF-1 cells with or without HCMV infection were blocked with 10% FBS in PBS at 37°C for 1 hour, then stained for viral gene products with primary antibody for 1 hour, followed by secondary antibody for 30 minutes each at 37°C in the dark. All slides were mounted in ProLong Gold anti-fade reagent with DAPI (Invitrogen, Grand Island, NY) and analyzed with an inverted AxioObserver fluorescence microscope (Carl Zeiss Microscopy, Oberkochen, Germany) mounted with a digital camera. Digital images were taken with AxioVision Rel 4.8 software (Carl Zeiss Microscopy).

RNA Preparation and Quantitative Real-Time Polymerase Chain Reactions (qRT-PCR). Two T75 flasks at 90% confluence containing $3x10^6$ MCF-7 cells each were treated with 100

ng/mL purified recombinant cmvIL-10 (R&D Systems) for 24 hours, or an equal amount of PBS as a vehicle control (Figure 7). The RNA was harvested from cells using the RNeasy Midi Kit (Qiagen, Germantown, MD) according to manufacturer's instructions and treated with RNase-Free DNase (Qiagen) to further remove any contaminating DNA from the samples. RNA was analyzed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) to measure concentration and purity. Using the RT^2 First Strand Kit (SABiosciences, Valencia, CA), cDNA was synthesized via reverse transcription from 1 μg of purified RNA. Polymerase chain reaction (PCR) component mixes were prepared using RT^2 SYBR Green Mastermix (SABiosciences) according to manufacturer's instructions, and 25 μL mix was dispensed into each well of the RT^2 Profiler PCR Human Tumor Metastasis Array (SABiosciences, PAHS-028ZD-2) (Figure 7). The plates were run using the CFX96 Real-Time System cycler (BioRad, Hercules, CA), adhering to a two-step cycling program. One 10-minute cycle at 95°C was followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. MyiQ software (BioRad) was used to analyze gene expression.

Enzyme-Linked Immunosorbent Assay (ELISA). Clear bottom 96-well plates were seeded with $5x10^3$ MCF-7 cells per well and treated with 0, 1, 10, or 100 ng/mL purified recombinant cmvIL-10 (R&D Systems) in triplicate. Cell supernatants were harvested and frozen at -20°C after 24, 48, 72, 96, 120, or 144 hours for subsequent analysis. Cell lysates were also collected from these plates by adding 100μL cell lysis buffer (150mM NaCl, 20 mM HEPES, 0.5% Triton X-100, 1mM $NaOV_4$, 1mM EDTA, 0.1% NaN_3) with 1:100 fresh protease inhibitor (Calbiochem, La Jolla, CA) onto the cells and administering a freeze-thaw cycle. Where indicated, MCF-7 cells were also treated with conditioned medium from HFF-1 cells infected

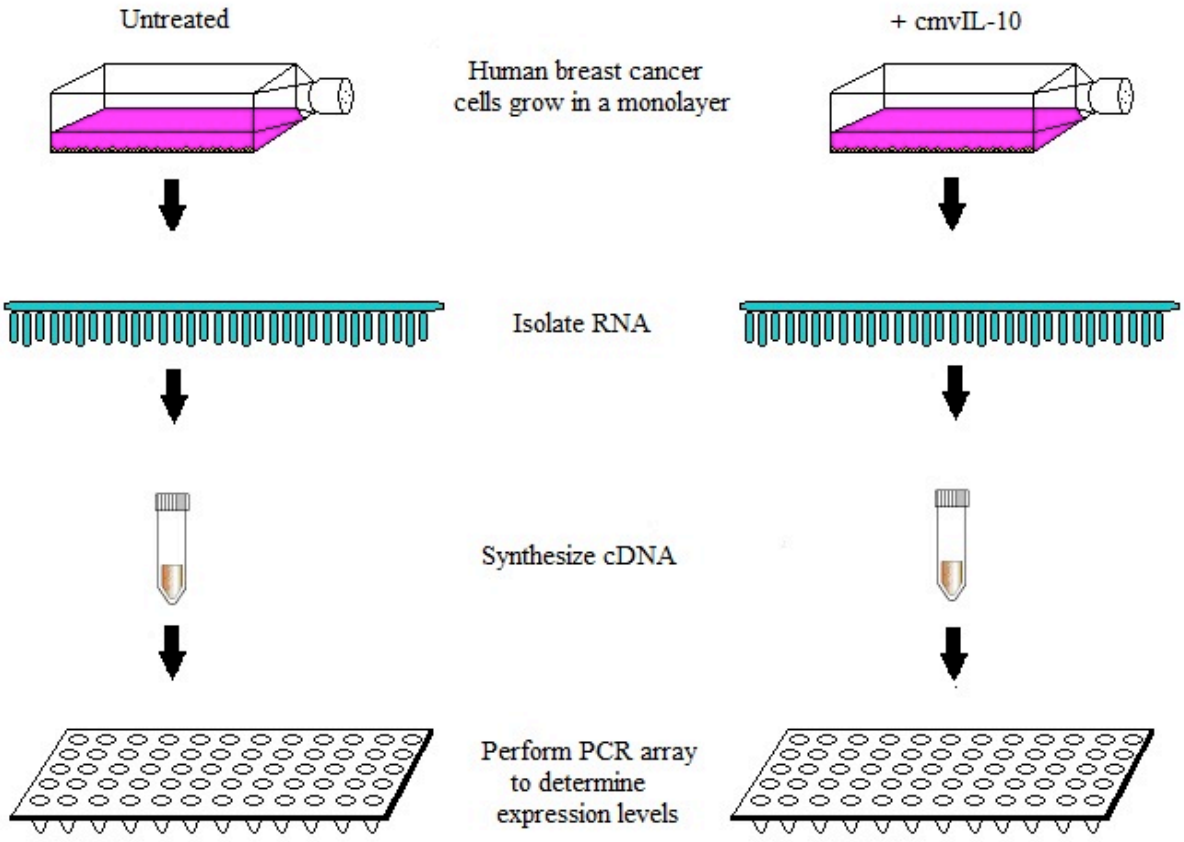

Figure 7. A schematic illustration of the qRT-PCR method. To compare gene expression related to metastasis of breast cancer cells in the presence or absence of purified recombinant cmvIL-10, RNA was isolated from cells, cDNA was synthesized, and plates were loaded with samples for PCR array.

with HCMV AD-169. MCF-7 cells were seeded as described above and grown in 1:1 complete media and conditioned medium from HFF-1 cells 5 days post infection exhibiting 100% CPE. Supernatant and cell lysate samples were collected and frozen as discussed above. Samples were then measured for MMP 2, MMP 3, MMP 7, MMP 9, MMP 10, human IL10, TGFβ, TNFα, IL-6, IL-1β protein levels by ELISA, performed according to manufacturer's instructions (R&D Systems Duo Sets). Samples of supernatant from HFF-1 cells in the presence or absence of HCMV infection were also evaluated by ELISA for the aforementioned proteins. Protein concentrations in each of the cell supernatant or cell lysate samples were determined by interpolation from a standard curve with an R^2 value greater than 0.99 using the Opsys MR plate reader and Revelation Quicklink 4.24 software (Dynex Technologies, Chantilly, VA).

Migration Assays. Cells were cultivated in serum free media in the presence or absence of 100 ng/mL cmvIL-10 for 24 hours. For migration assays, well filters (ThinCerts, 24 well, 8 μm pore, E & K Scientific, Santa Clara, CA) were loaded into 24-well plates using sterile tweezers to create Boyden Chambers (Figure 8). Cells were harvested via scraping, washed, and resuspended in fresh serum free media. A total of $2x10^5$ cells in a volume of 100 μL in the presence or absence of 100 ng/mL cmvIL-10 were dispensed into each upper chamber. Lower chambers were loaded with 600 μL media with 1% FBS and 100 ng/mL cmvIL-10 was added to media in the lower chambers of wells where indicated (Figure 8). The plates were incubated at 37°C for 4 hours, and then cells were harvested from the lower chamber by dipping the filter in the lower chamber media 10 times and rinsing the bottom of the filter twice using lower chamber media. Media and cells that were adhered to the outside of the filter were removed by pipet, and then the lower chamber well media was collected and placed into a microfuge tube and centrifuged at 1000 rpm for 5 minutes. All but 100 μL of media was removed from the

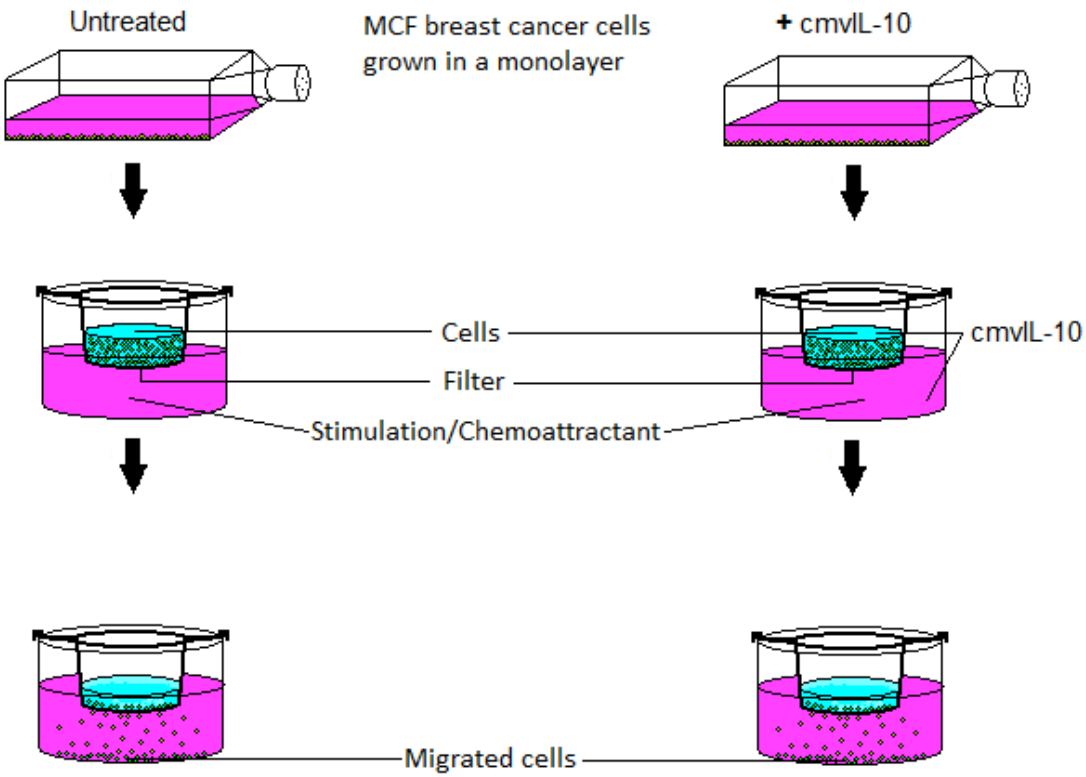

Figure 8. Boyden chamber assay to investigate cell motility. The upper chambers contain breast cancer cells suspended in serum free media in the presence or absence of cmvIL-10. The lower chambers contain 1% FBS in media in the presence or absence of cmvIL-10. Migration of breast cancer cells from the upper chamber to the lower chamber was quantified by harvesting cells from the lower chamber after 4 hours of incubation and measuring relative light units with the Cell Titre Glo Assay.

microfuge tube, in which the cell pellets were resuspended. The samples were placed into a white clear bottom assay plate and cell quantity was measured using the Cell Titre Glo Assay according to the manufacturer's instructions. The plates were read with a GloMax luminometer (Promega) using GlowMax software to measure relative light units.

Results

I. Human IL-10 Receptor Is Present on the Surface of Breast Cancer Cells

This research project explores the alteration of the cellular activities of human breast cancer cells when placed in the presence of a viral cytokine, cmvIL-10. To determine whether cmvIL-10 has a role in modulating breast cancer metastasis, we examined the availability of the human IL-10 cellular receptor on the surface of MCF-7 breast cancer cells. Flow cytometry was performed on MCF-7 cells that had been cultivated in the absence of serum and then stained with fluorochrome-conjugated IL-10R α and β antibodies. Figure 9 illustrates the presence of both chains of the hIL-10 receptor on the surface of the cancer cells, as evidenced by right shifted fluorescence compared to isotype control antibodies.

In addition to flow cytometry, we employed immunofluorescence microscopy to demonstrate the availability of hIL-10R for binding with cmvIL-10 on the surface of MCF-7 breast cancer cells and to visualize IL-10R distribution throughout the cell. Cells were grown on glass cover slips, fixed, permeabilized and labeled with an antibody specific for hIL-10Rα. The image in Figure 10 illustrates hIL-10Rα is distributed in a discrete punctate fashion throughout the MCF-7 cells. Cell nuclei are labeled with DAPI in blue and the cytokine receptor hIL-10Rα is labeled in red. The receptor is present inside and on the surface of the breast cancer cells, including extending along cellular appendages. The microscopy results, in conjunction with the flow cytometry results, demonstrate the abundant availability of human IL-10 receptor on the surface of MCF-7 breast cancer cells.

II. CmvIL-10 Changes the Transcriptional Expression of Tumor Metastasis-Related Genes

The complete alteration of cellular activity due to the binding and signaling of cmvIL-10 is not fully defined. Considering the connection between HCMV and cancer progression, we

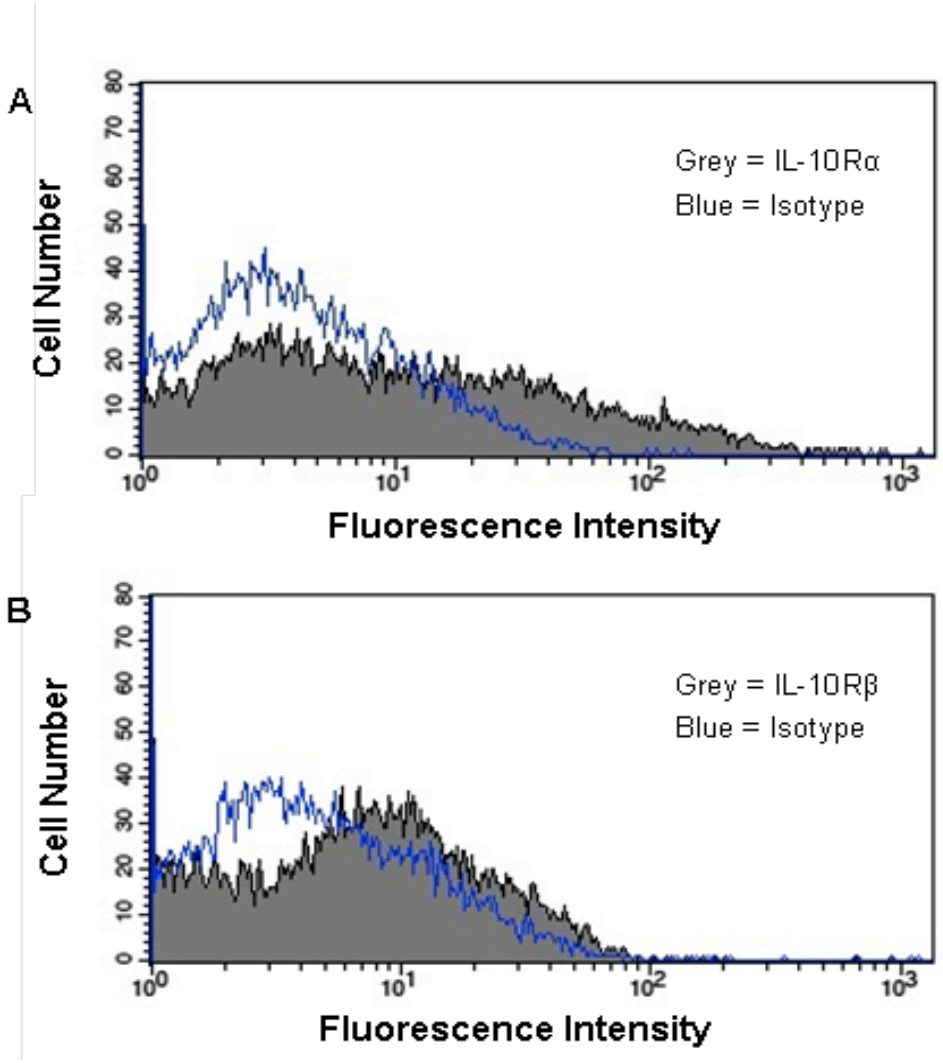

Figure 9. Flow cytometry shows the Human IL-10 Receptor is present on the surface of MCF-7 breast cancer cells. Flow cytometry was performed on serum-starved MCF-7 cells in order to confirm the expression of hIL-10R on the surface of the cells. In the upper panel A, the blue line in the histogram denotes the goat IgG isotype, and the grey shaded area shifted to the right illustrates the presence of the fluorochrome-conjugated goat anti-IL-10Rα antibody detected on the surface of the cells. In the lower panel B, the blue line in the histogram denotes the mouse IgG_1 isotype, and the grey area shifted to the right illustrates the presence of the fluorochrome-conjugated goat anti-IL-10Rβ antibody detected on the surface of the cells.

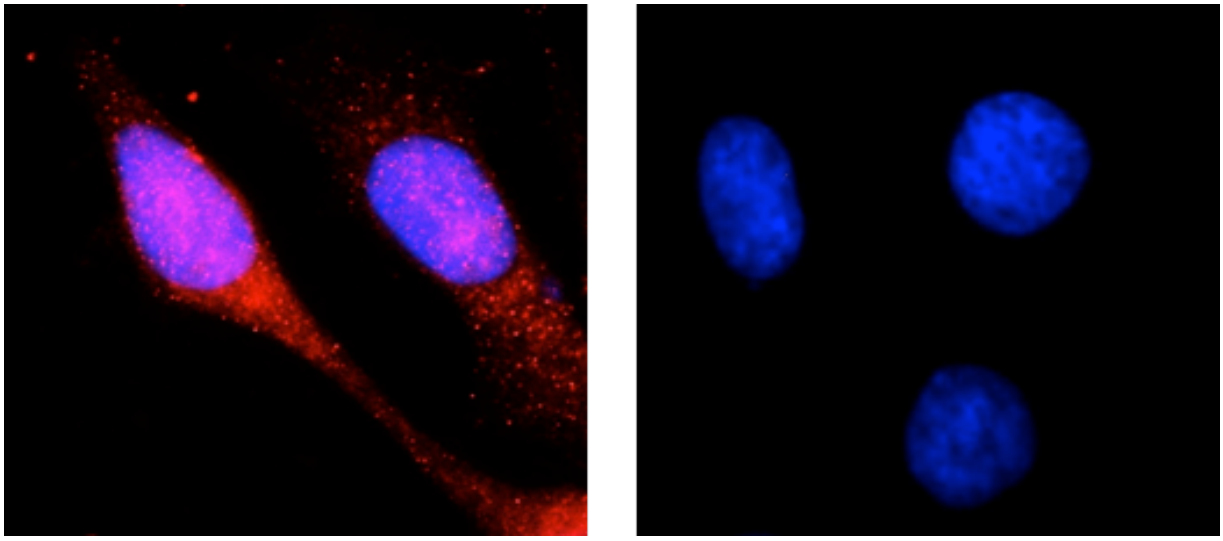

Figure 10. Immunofluorescence microscopy reveals the Human IL-10α Receptor on MCF-7 breast cancer cells. Immunofluorescent staining with rabbit anti-hIL-10Rα followed by goat anti-rabbit-TRITC was performed to visualize the cytokine receptor. In the image of the left, the cell nuclei are labeled blue with DAPI and the human IL-10 receptor is shown in red. The image on the right is stained with DAPI and the secondary antibody goat anti-rabbit-TRITC as a control.

investigated the impact of purified, recombinant cmvIL-10 on transcription of genes associated with tumor metastasis in breast cancer cells. We conducted gene expression analysis using Human Tumor Metastasis RT^2 Profiler PCR Arrays (SA Biosciences, Frederick, Maryland), which contain primers for 84 genes associated with human tumor metastasis (Table 2 in Appendix). First, highly purified RNA was isolated from MCF-7 breast cancer cells that were cultured in the presence or absence of cmvIL-10, cDNA was synthesized from the prepared RNA via reverse transcription, and the samples were applied to the microarrays. The microarray plates were run using a BioRad CFX96 Real-Time PCR machine and a change in gene expression was determined by comparing the amount of transcription of the genes of interest in cells treated with cmvIL-10 compared to the amount of transcription of the genes of interest in cells not treated with cmvIL-10. Figure 11 summarizes the results of 3 experiments and shows that treatment with cmvIL-10 changes gene expression of MCF-7 breast cancer cells. A fold change of more than one is interpreted as an up-regulation of the gene of interest, and a fold change of less than one is interpreted as a down-regulation of the gene of interest. Although differences between the means were not statistically significant ($p > 0.05$, student's t-test), Figure 11 indicates, in green, five genes which were up-regulated more than 2-fold due to treatment with cmvIL-10 for both 24 and 48 hours, including FAT1, ITGB3, MCAM, SET, and SMAD2. Two additional genes indicated in green, SSTR2, RB1, were also up-regulated more than 2-fold by cmvIL-10 after 48 hours of treatment. Moreover, Figure 11 indicates 4 other genes, shown in yellow, were up-regulated after both 24 hours and 48 hours treatment with cmvIL-10 more than 1.5-fold, DENR, EPHB2, PNN, RPSA. These results illustrate a trend of enduring up-regulation due to treatment with cmvIL-10 for the genes mentioned. Furthermore, 6 genes, shown in yellow, were up-

Gene	Gene Description	24 Hours	48 Hours
FGFR4	Fibroblast growth factor receptor 4	0.533	0.677
CST7	Cystatin F (leukocystatin)	0.573	0.753
RB1	Retinoblastoma 1	0.593	2.413
IL1B	Interleukin 1, beta	0.637	1.137
FN1	Fibronectin 1	0.643	0.983
NF2	Neurofibromin 2 (merlin)	0.647	0.887
SYK	Spleen tyrosine kinase	0.647	0.847
CHD4	Chromodomain helicase DNA binding protein 4	0.650	0.847
MYCL1	V-myc myelocytomatosis viral oncogene homolog 1	0.663	1.287
EWSR1	Ewing sarcoma breakpoint region 1	0.683	0.813
MDM2	Mdm2 p53 binding protein homolog (mouse)	0.683	0.747
SRC	V-src sarcoma viral oncogene homolog	0.687	0.967
MTSS1	Metastasis suppressor 1	0.690	1.030
CD82	CD82 molecule	0.700	1.077
CXCL12	Chemokine (C-X-C motif) ligand 12	0.710	0.890
APC	Adenomatous polyposis coli	0.730	1.267
FXYD5	FXYD domain containing ion transport regulator 5	0.740	1.043
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	0.757	0.873
NR4A3	Nuclear receptor subfamily 4, group A, member 3	0.757	0.953
MGAT5	Mannosyl glycoprotein acetyl-glucosaminyltransferase	0.763	0.920
CD44	CD44 molecule (Indian blood group)	0.767	0.917
GNRH1	Gonadotropin-releasing hormone 1	0.767	0.870
MTA1	Metastasis associated 1	0.810	1.040
VEGFA	Vascular endothelial growth factor A	0.863	1.123
CTBP1	C-terminal binding protein 1	0.873	1.323
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.917	1.067
MET	Met proto-oncogene (hepatocyte GF receptor)	0.923	1.153
KISS1	KiSS-1 metastasis-suppressor	0.940	1.287
SMAD4	SMAD family member 4	0.940	0.837
IL18	Interleukin 18 (interferon-gamma-inducing factor)	0.947	1.033
MMP11	Matrix metallopeptidase 11 (stromelysin 3)	0.960	1.007
TGFB1	Transforming growth factor, beta 1	0.973	1.467
TNFSF10	Tumor necrosis factor (ligand) superfamily	0.977	1.133
TP53	Tumor protein p53	0.977	1.427
METAP2	Methionyl aminopeptidase 2	0.993	0.933
ETV4	Ets variant 4	1.057	1.293
TIMP2	TIMP metallopeptidase inhibitor 2	1.060	1.267
BRMS1	Breast cancer metastasis suppressor 1	1.067	1.490
NME1	Non-metastatic cells 1, protein (NM23A)	1.070	1.127
TCF20	Transcription factor 20 (AR1)	1.090	1.430
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	1.093	1.230
MMP9	Matrix metallopeptidase 9 (gelatinase B)	1.103	1.187
CTNNA1	Catenin (cadherin-associated protein), alpha 1	1.117	1.403
NME4	Non-metastatic cells 4	1.137	1.263
CXCR4	Chemokine (C-X-C motif) receptor 4	1.153	1.520
FLT4	Fms-related tyrosine kinase 4	1.153	1.313
ITGA7	Integrin, alpha 7	1.187	1.050
HPSE	Heparanase	1.203	1.063
CCL7	Chemokine (C-C motif) ligand 7	1.217	1.077
CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	1.217	1.077
CDKN2A	Cyclin-dependent kinase inhibitor 2A	1.217	1.077
CTSL1	Cathepsin L1	1.217	1.077
CXCR2	Chemokine (C-X-C motif) receptor 2	1.217	1.077
HGF	Hepatocyte growth factor (hepapoietin A)	1.217	1.077
IGF1	Insulin-like growth factor 1 (somatomedin C)	1.217	1.077
KISS1R	KISS1 receptor	1.217	1.700
MMP13	Matrix metallopeptidase 13 (collagenase 3)	1.217	1.077
MMP3	Matrix metallopeptidase 3 (stromelysin 1)	1.217	1.077
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	1.217	1.077
RORB	RAR-related orphan receptor B	1.217	1.077
TIMP3	TIMP metallopeptidase inhibitor 3	1.217	1.077
TIMP4	TIMP metallopeptidase inhibitor 4	1.217	1.077
TRPM1	Transient receptor potential cation channel	1.217	1.077
TSHR	Thyroid stimulating hormone receptor	1.217	1.077
COL4A2	Collagen, type IV, alpha 2	1.250	0.993
NME2	Non-metastatic cells 2, protein (NM23B)	1.250	1.160
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	1.257	1.390
SSTR2	Somatostatin receptor 2	1.260	2.373
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	1.277	1.407
MMP2	Matrix metallopeptidase 2 (gelatinase A)	1.283	1.077
CTSK	Cathepsin K	1.323	1.547
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	1.343	1.310
MYC	V-myc myelocytomatosis viral oncogene homolog	1.397	1.870
PTEN	Phosphatase and tensin homolog	1.433	1.533
PLAUR	Plasminogen activator, urokinase receptor	1.440	1.723
EPHB2	EPH receptor B2	1.510	1.803
PNN	Pinin, desmosome associated protein	1.783	1.820
RPSA	Ribosomal protein SA	1.827	1.747
DENR	Density-regulated protein	1.840	1.710
FAT1	FAT tumor suppressor homolog 1 (Drosophila)	2.213	2.640
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa)	2.287	4.853
SMAD2	SMAD family member 2	2.440	2.573
SET	SET nuclear oncogene	2.483	2.307
MCAM	Melanoma cell adhesion molecule	3.243	3.193

> 2-fold Down-Regulation No Significant Change 1.5 – 2-fold Up-Regulation
1.5 – 2-fold Down-Regulation > 2-fold Up-Regulation

Figure 11. Fold change in tumor metastasis-related gene expression after treatment with cmvIL-10 for 24 and 48 hours. MCF-7 breast cancer cells in the presence or absence of cmvIL-10 for 24 and 48 hours were analyzed for expression of genes related to tumor metastasis by qRT-PCR arrays. The fold change represents the ratio between the gene expression in the cmvIL-10 treated cells and the untreated control cells, averaged from 3 separate experiments.

regulated more than 1.5 fold after a full 48 hours treatment with cmvIL-10, including CTSK, CXCR4, KISS1R, MYC, PLAUR, and PTEN.

Treatment with cmvIL-10 also down-regulated MCF-7 gene expression, but only modestly, as down-regulation was observed only after 24 hours of treatment, and no genes were down-regulated more than 2-fold. A fold change below 0.66 represents a greater than 1.5-fold down-regulation in gene expression. Eight genes, shown in orange in Figure 11, were down-regulated between 1.5- and 2-fold due to treatment with cmvIL-10 for 24 hours, including CHD4, CST7, GFR4, IL1B, NF2, RB1, RN1, and SKY. Noteworthy is RB1, which is the only gene to have been distinctly down-regulated at 24 hours of treatment with cmvIL-10 and then significantly up-regulated after 48 hours of treatment with cmvIL-10.

To further analyze qRT-PCR results and discern a more clear depiction of the role of cmvIL-10 in gene expression alteration, we sorted the genes into functional groups and removed outliers, which are defined as fold changes more than 3 times different than the average of the other two fold changes of the same treatment condition. Results from genes associated with cell adhesion were grouped together. Cell adhesion is important for tissue integrity and stability. Cancer cells that lose cell adhesion characteristics may be more likely to undergo invasion and develop a metastatic tumor in another part of the body. Analysis of the qRT-PCR results with a focus on genes that encode for gene proteins related to cell adhesion reveals that cmvIL-10 may promote a reduction in cell adhesion (Figure 12). MCAM, or melanoma cell adhesion molecule, also known as CD146 and MUC18, is a gene associated with cell migration and angiogenesis. MCAM was up-regulated nearly 8-fold after treatment with cmvIL-10 at both 24 and 48 hour time points. This calculation is representative of two experiments with similar results demonstrating the significant up-regulation of MCAM due to treatment with cmvIL-10 for 24

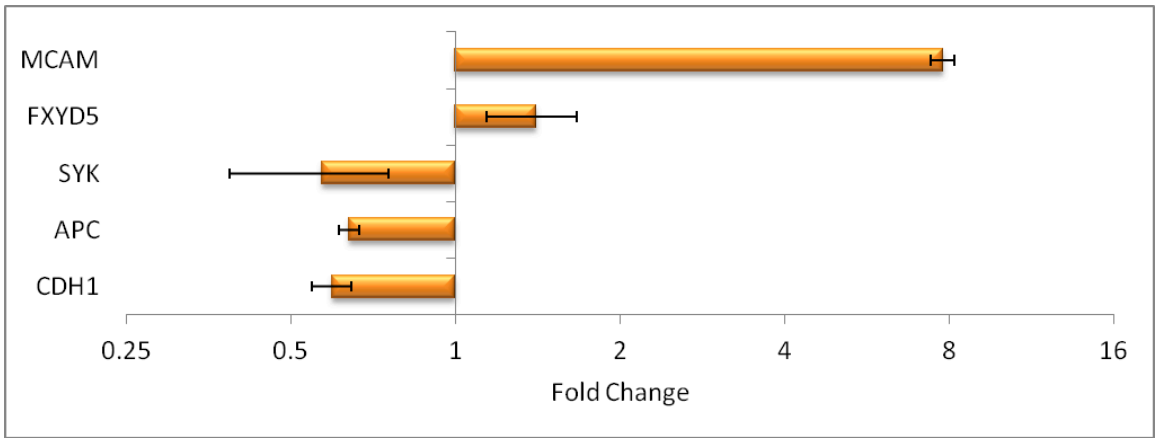

Figure 12. Depiction of the fold change in adhesion-associated gene expression after treatment with cmvIL-10. MCF-7 breast cancer cells in the presence or absence of cmvIL-10 for 24 hours were analyzed for expression of genes related to cell adhesion by qRT-PCR array. The fold change represents the ratio between the gene expression in the treated cells and the control cells. MCAM and FXYD5 are correlated with increased invasion, while SYK, APC, and CDH1 suppress invasion. Overall, the modulation of adhesion-associated gene expression by cmvIL-10 demonstrates a shift toward a transcript profile favoring more metastasis. Error bars represent the range of fold-change values.

hours. Additionally, treatment with cmvIL-10 up-regulated FXYD5, or FXYD domain containing ion transport regulator 5, also known as dysadherin, a gene involved in the reduction of cell adhesion. These two genes exhibited an up-regulation in expression due to treatment with cmvIL-10, which may result in less cell adhesion and lead to a more invasive phenotype.

Treatment with cmvIL-10 also down-regulated genes associated with cell adhesion. Figure 12 depicts the down-regulation of APC, CDH1, and SYK, cell adhesion genes which are associated with tumor suppression. Tumor suppressor genes are involved in repressing the cell cycle and promoting apoptosis. A loss of function of tumor suppressor genes may lead to unregulated cell growth and increased metastasis due to amplified cell proliferation, increased angiogenesis, elevated cell detachment and more robust migration. We show that cmvIL-10 alters the gene expression profile of genes related to cell adhesion, manipulating cells to produce less tumor suppressor gene transcripts, which may cause a phenotypic change favoring metastasis.

Another group of genes analyzed were the genes encoding for MMPs. MMPs degrade the extracellular matrix and permit tumor cells access to the vascular and lymphatic systems, allowing cancer cells to disseminate. Figure 13 depicts the comparison of gene expression of cells treated with cmvIL-10 for 24 hours and untreated control cells. Fold changes were calculated by removing outliers and taking the average of two similar ratios. All MMPs genes present on the array were up-regulated in MCF-7 cells exposed to cmvIL-10 for 24 hours. Gene expression of MMP2, MMP3, MMP 11, MMP13 was up-regulated about 1.4-fold and MMP7 was up-regulated 1.6-fold. MMP9 and MMP10 were up-regulated the most due to cmvIL-10 treatment, 2.4-fold and 4.8-fold, respectively. There was no remarkable change in TIMP gene expression to counteract the increase in MMP gene expression, with TIMP2, TIMP3, and TIMP4

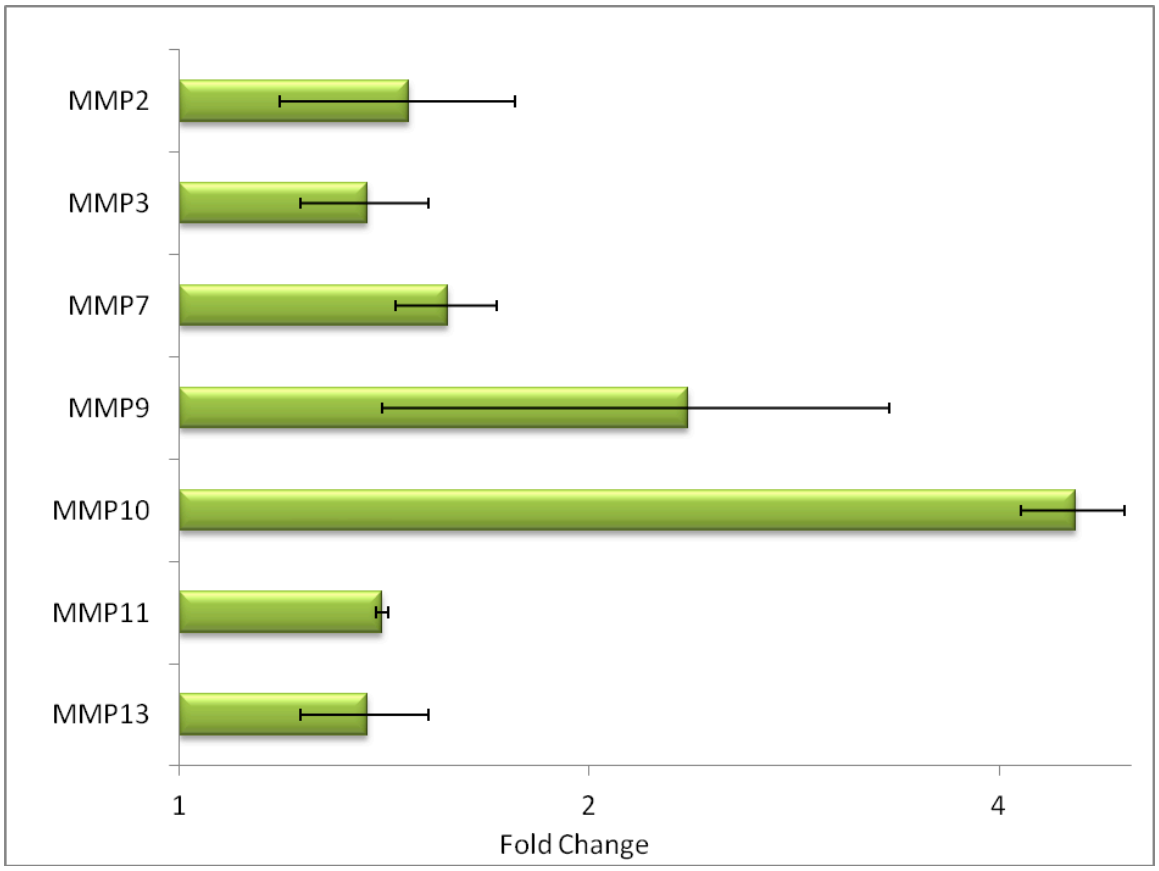

Figure 13. Depiction of the fold change in MMP gene expression after treatment with cmvIL-10. MCF-7 breast cancer cells in the presence or absence of cmvIL-10 for 24 hours were analyzed for expression of genes encoding for MMPs by qRT-PCR. The fold change represents the ratio between the gene expression in the treated cells and the control cells. All MMPs were up-regulated due to treatment with cmvIL-10. MMP9 was up-regulated 2.4-fold and MMP10 was up-regulated 4.6-fold. This shift in gene expression may cause cells to secrete more MMPs, further degrading the extracellular matrix and permitting dissemination. Error bars represent the range of fold-change values.

all exhibiting average fold-changes between 1 and 1.2. These results indicate that cmvIL-10 increases the transcription of genes encoding for MMPs, which may elevate secretion of MMP protein levels and promote metastasis.

III. Examination of the Secretome of Breast Cancer Cells Treated with CmvIL-10

To investigate if the changes in gene expression observed by qRT-PCR correlate with an increase in protein levels, the presence of MMPs in supernatants from MCF-7 cells treated with purified recombinant cmvIL-10 was examined. Supernatants were collected and the levels of MMP2, MMP3, MMP7, MMP9, and MMP10 were determined by sandwich ELISA. While total secreted MMP levels were expected to be higher in breast cancer cells due to the observed increase in MMP gene expression, no MMP protein was detected in cell supernatants following cmvIL-10 treatment. ELISAs were also conducted measuring the levels of TIMP1 in MCF-7 cell supernatant to determine if TIMP1 was present and might be interfering with the detection of MMP proteins. Abundant TIMP1 was detected, unlike MMPs, yet no change in TIMP1 levels was observed due to the treatment of cmvIL-10. Figure 14 illustrates the presence of TIMP1 proteins in MCF-7 cell supernatant, and a lack in change of TIMP1 levels due to treatment with cmvIL-10. These data support the qRT-PCR data showing a minimal change in TIMP gene expression due to treatment with cmvIL-10.

While an increase in MMP gene expression was observed by qRT-PCR, it is possible that MMP protein levels are elevated in breast cancer cells in the presence of cmvIL-10, but the proteins are not being secreted by the cells. MMPs are often cell-associated, and these membrane-type (MT)-MMPs would not be secreted by the cell into the supernatant, but remain tethered to the cell membrane. To investigate if the change in MMP gene expression could be

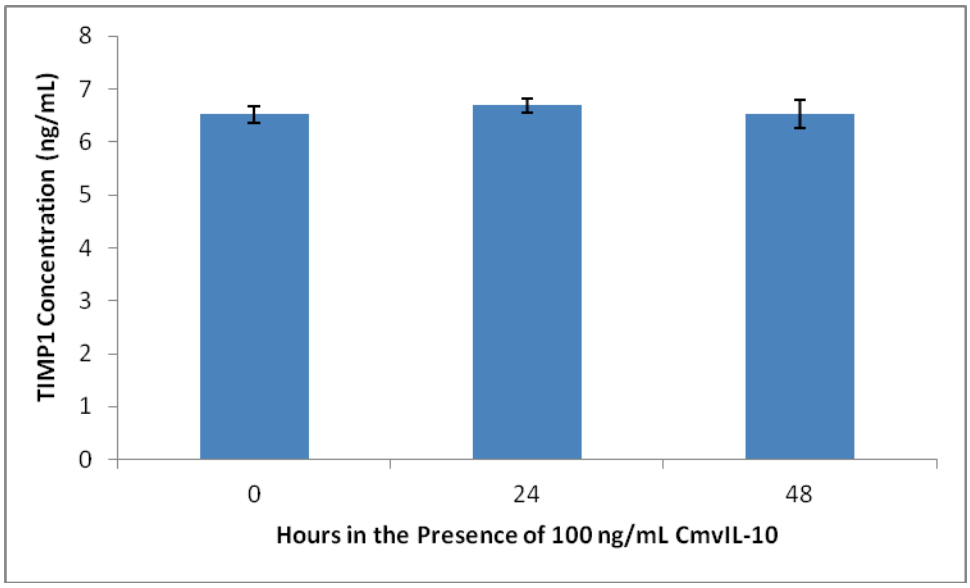

Figure 14. Extracellular TIMP1 concentration is not affected by cmvIL-10. MCF-7 breast cancer cells in the presence or absence of cmvIL-10 were analyzed for TIMP1 protein levels by ELISA. The concentration of TIMP1 was not altered due to treatment with the viral cytokine cmvIL-10. These results support the qRT-PCR results demonstrating no change in gene expression was observed for the TIMP genes investigated. Error bars represent the range of concentrations measured for each condition.

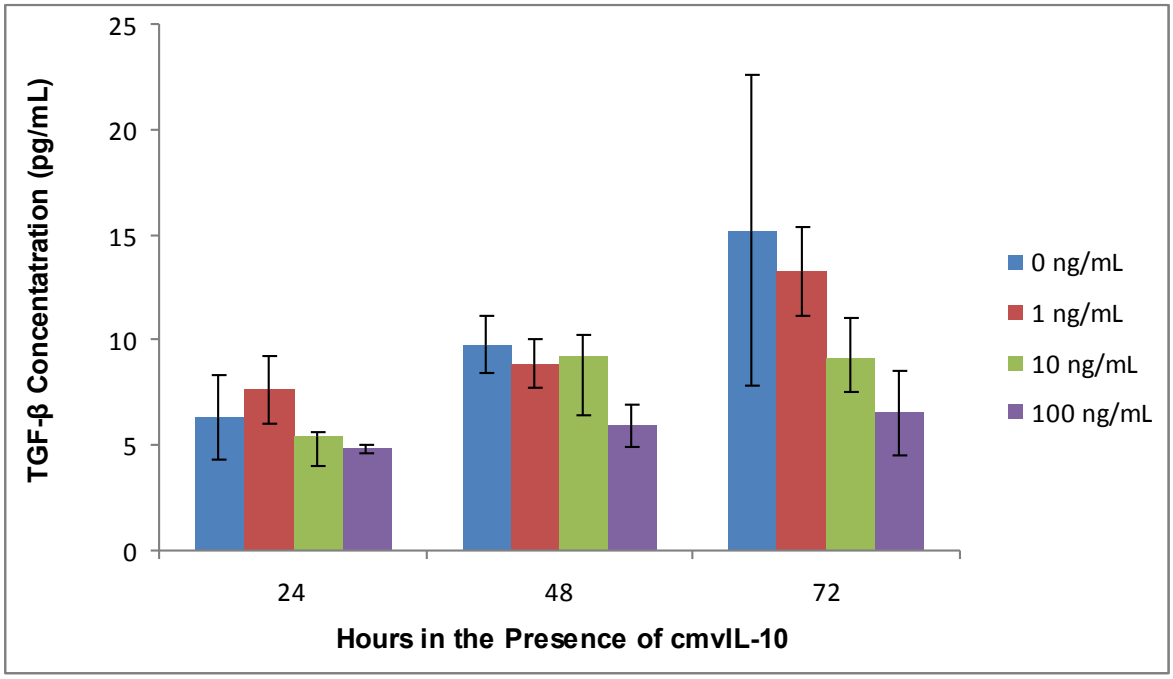

Figure 15. Extracellular TGF-β concentration is decreased by cmvIL-10. MCF-7 breast cancer cells in the presence or absence of cmvIL-10 were analyzed for TGF-β protein levels by ELISA. The concentration of TGF-β was decreased by the viral cytokine cmvIL-10, most substantially after 72 hours of treatment. Error bars represent the range of concentrations measured for each condition.

translated into an increase in MT-MMPs, cell lystates were collected and screened for MMP9 and MMP10. However, no MMP9 or MMP10 were detected by ELISA from the cell lysate samples. Future investigations of MMP2, MMP3 and MMP7 may uncover MMP protein in cell lysates from MCF-7 cells treated with cmvIL-10.

Furthermore, the secretome of MCF-7 breast cancer cells treated with cmvIL-10 was also examined for other proteins associated with cancer progression by ELISA, including TGF-β, TNF-α and interleukins IL-1β and IL-6. Figure 15 shows that TGF-β protein levels were found to decrease due to treatment with cmvIL-10, despite only minimal alterations at the geneexpression level. While TGF-β is often largely considered a mitogenic protein, it also contributes to facilitating apoptosis. Therefore, cmvIL-10 could promote tumor progression by decreasing TGF-β, which may lead to less apoptosis and increased viability of breast cancer cells. Lastly, no TNF-α, IL-1β, or IL-6 proteins were detected in cell supernatants.

IV. CmvIL-10 Promotes Migration

Cellular activity depends on an enormous variety of factors. One cell activity that is vital for metastasis is the ability of cells to be motile. Using Boyden chambers, we investigated the migration capability of breast cancer cells toward fetal bovine serum, which contains a variety of growth factors and chemotactic agents, in the presence or absence of cmvIL-10. We determined the concentration of FBS that provided the most motility from the upper to the lower chamber. MCF-7 cells were harvested by scraping an 80% confluent monolayer and $2x10^5$ cells were placed in the upper chamber of each well in 100 μL serum free media. In the lower chamber, medium containing varying amounts of FBS was plated. After 4 hours incubation at 37°C, cells were harvested and relative light units were measured using the Cell Titer Glo Assay. Figure 16 depicts that MCF-7 cells migrate robustly toward 1% FBS in lower chambers. To investigate the

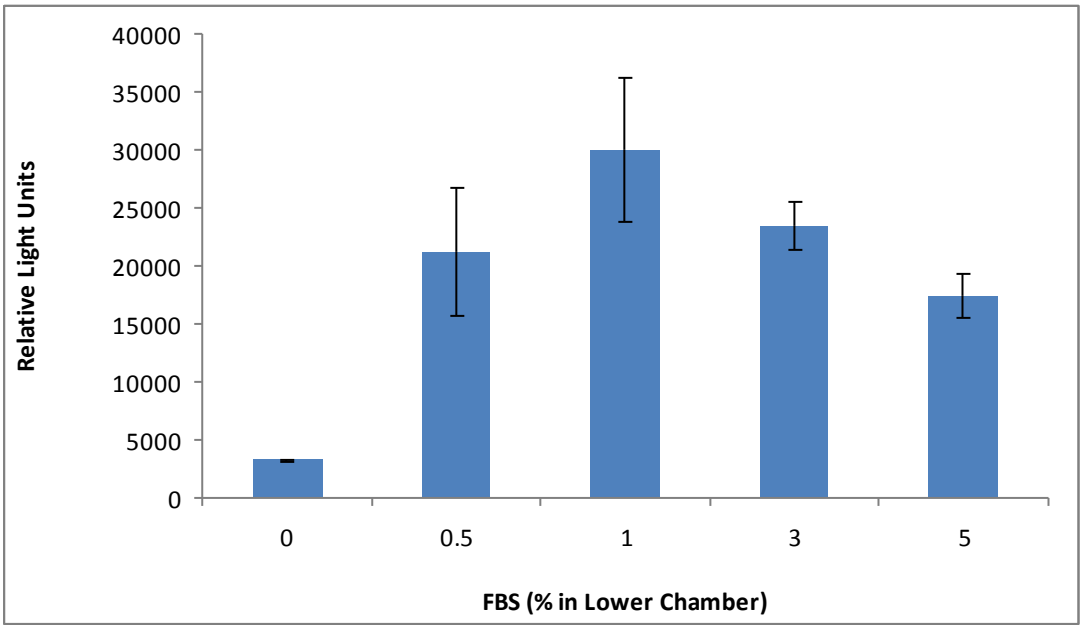

Figure 16. Breast cancer cells migrate robustly toward 1% FBS. MCF-7 breast cancer cells were placed in the upper portion of the Boyden chamber in serum free media and migrated toward media with different concentrations of FBS in the lower chambers. Error bars represent the range of RLUs measured for each condition. N = 3.

ability of cmvIL-10 to modulate this migratory action, assays were constructed so that all lower chambers had 1% FBS.

To determine optimal placement of cmvIL-10 in the Boyden chamber, 100 ng/mL cmvIL-10 was placed in the serum free media upper chamber, in the lower chamber with 1% FBS, in both chambers, or in neither. Figure 17 shows that the most active migration is observed when cmvIL-10 is in both the upper and lower chambers. Fold changes were calculated by comparing migration toward serum free media in the lower chamber (blue bars) and migration toward 1% FBS in the lower chambers (red bars) with cmvIL-10 in the same location. While migration with cmvIL-10 in both the upper and lower chambers did not have the largest fold change, it was high and had the least error. We decided to pursue this condition in future assays. Different concentrations of cmvIL-10 were explored to investigate which concentration promoted migration the most. Figure 18 illustrates that MCF-7 cells in the presence of 1 ng/mL cmvIL-10 migrate toward cmvIL-10 and 1% FBS more than any other condition. These results indicate that breast cancer cells in the presence of cmvIL-10 migrate 13-fold more than cells not treated with the viral cytokine. This represents a 138% increase in migration compared to untreated breast cancer cells, calculated by determining the percent of the ratio of the average RLUs measured from migrated cells treated or untreated with cmvIL-10. The results demonstrate that cmvIL-10 impacts breast cancer cell activity by increasing cell migration, which may contribute to breast cancer progression by promoting metastasis in the body.

V. Examination of the Secretome of HFF-1 Cells Infected with HCMV

HCMV infected cells may secrete many factors other than cmvIL-10 that could influence tumor progression. We considered it possible that breast cancer cells, upon exposure to supernatants from nearby virus infected cells, may secrete more proteins like MMPs after

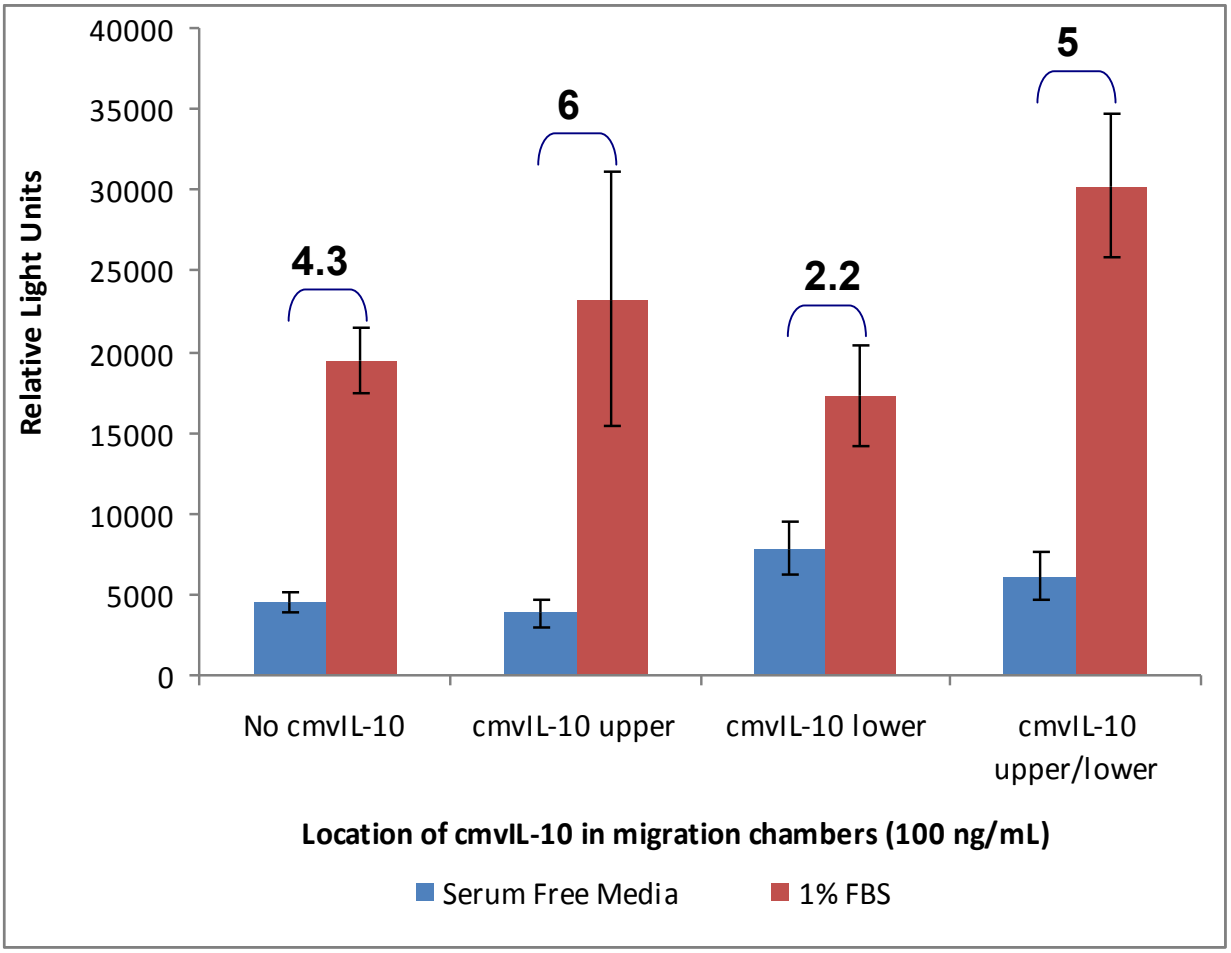

Figure 17. Breast cancer cells migrate robustly when cmvIL-10 is in both upper and lower chambers. MCF-7 breast cancer cells were placed in the upper portion of the Boyden chamber in serum free media and migrated toward media with 1% FBS in the lower chambers (shown in red). Control chambers were established that contained cells in the same conditions except they migrated toward serum free media (shown in blue). 100 ng/mL cmvIL-10 was dispensed in the upper chamber, lower chamber, both chambers, or neither chamber. Fold changes were calculated by comparing the cell migration toward the media with 1% FBS in the lower chambers, and are indicated above each pair of bars in the graph. Error bars represent the range of RLUs measured for each condition. N = 3.

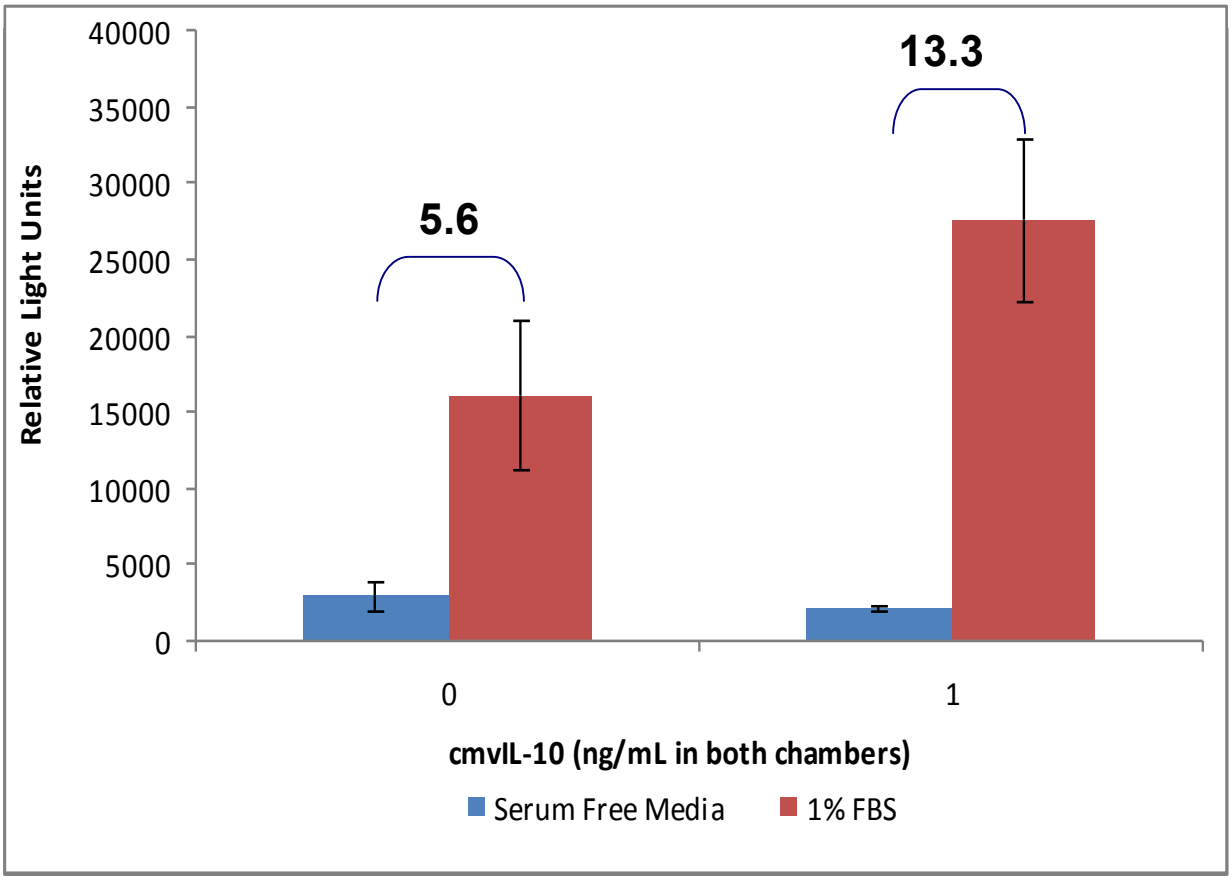

Figure 18. Breast cancer cells migrate more actively with 1 ng/mL cmvIL-10. MCF-7 breast cancer cells were placed in the upper portion of the Boyden chamber in serum free media and different concentrations of cmvIL-10 . Cells migrated toward media with 1% FBS in the lower chambers and the same concentration of cmvIL-10 as the upper chamber (red bars). Fold changes were calculated by comparing migration toward serum free media in the lower chamber with cmvIL-10 in the same location (blue bars) as the migration being measured toward 1% FBS in the lower chambers (red bars). Cells migrated most actively in chambers containing 1 ng/mL cmvIL-10, exhibiting a fold-change of 13.3, which is 138% more migration than cells not treated with cmvIL-10. Error bars represent the range of RLUs measured for each condition. N = 3.

receiving signals from HCMV infected cells. Additionally, it is possible that cmvIL-10 may cooperate with another human or viral protein to induce changes to promote metastasis of breast cancer cells. To explore these possibilities, it was important to first characterize the proteins present in the supernatant of HCMV infected cells in order to deduce how HCMV infection contributes to tumor progression.

Since HCMV infection is very selective in cell culture, human foreskin fibroblasts (HFF-1) were grown to confluency in a monolayer and subsequently infected with HCMV strain AD-169. Cytopathic effects were observed, including enlarged cells, retraction of cytoplasmic extensions, and irregular cell margins. Immunofluorescence was performed to confirm HCMV infection by staining for viral proteins IE1, US27, and US28. Figure 19 depicts images of uninfected HFF-1 cells on the left and images of HFF-1 cells infected with HCMV on the right. Cellular nuclei were stained with DAPI and can be observed in blue. The Immediate Early 1 gene product is stained in green and is co-localized in the nucleus of infected cells. The US27 viral gene product (green) is depicted in its customary distribution localized primarily on one side of the nucleus of infected cells, compared to no US27 protein present on uninfected cells. US28 protein is shown in a red punctate pattern in the HCMV infected cells, compared to a lack of US28 in uninfected cells.

After confirming infection of HFF-1 cells with HCMV, we examined the secretome of infected cells. HFF-1 cells were plated at $5x10^3$ cells per well and grown to 90% confluency. Wells were infected with equal amounts of isolated HCMV or PBS vehicle control. CPE was observed to be 10%, 30%, 80% at 24, 48 and 72 hours respectively when supernatants were collected and frozen at -20°C. Supernatants of HFF-1 cells infected or uninfected with HCMV were measured for protein levels by ELISA. MMP3 levels were shown to remain relatively

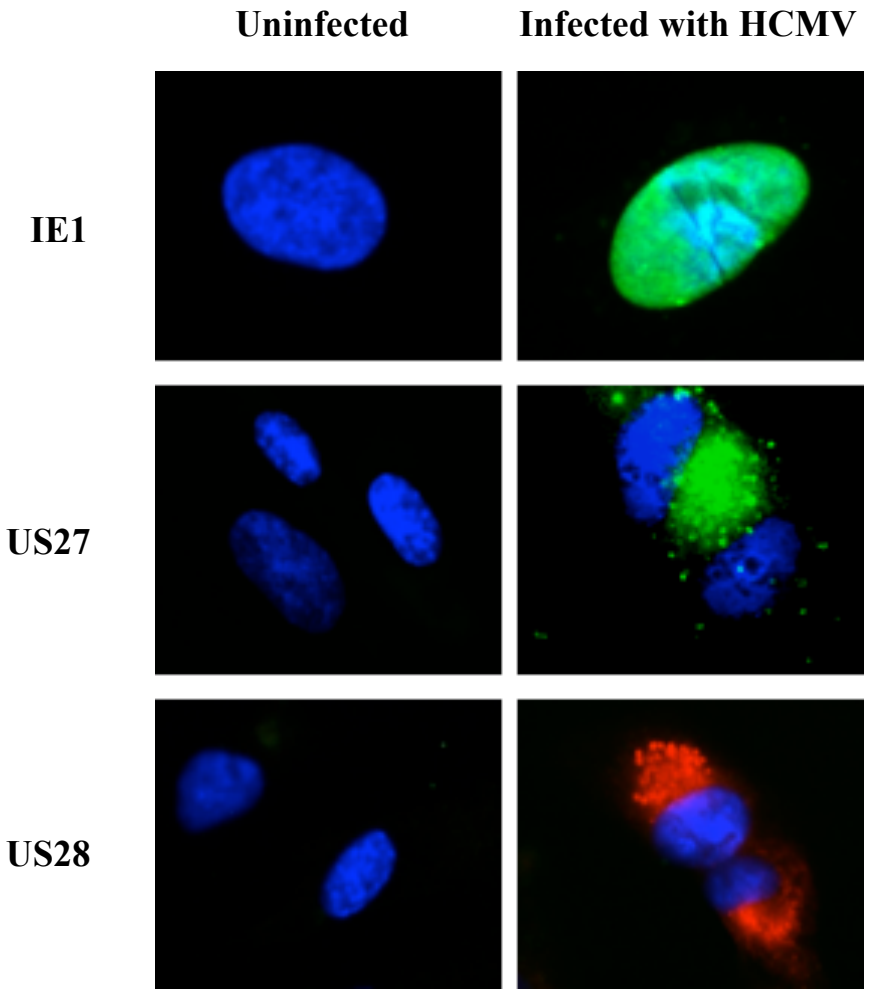

Figure 19. HFF-1 cells were infected with HCMV AD169 and stained for viral proteins IE1, US27 and US28. Immunofluorescence imaging shows cell nuclei stained with DAPI in blue. Uninfected HFF-1 cells are depicted in the left and HFF-1 cells infected with HCMV are on the right. A mouse anti-Immediate Early 1 antibody followed by goat anti-mouse-FITC stained the viral gene product IE1 green. A mouse anti-US27 antibody followed by goat anti-mouse-FITC stained the viral gene product US27 green also. A goat anti-US28 antibody followed by donkey anti-goat-TRITC stained the viral gene product US28 red.

constant over the course of the infection, and were observed at lower levels than uninfected HFF-1 cells (Figure 20). The concentration of MMP3 was lower in infected cells than uninfected cells at all time points post infection. The lower levels of MMP3 observed may have been due to the mortality of HFF-1 cells due to viral infection. Notably, the levels of MMP3 cells did not diminish over the course of the infection, despite fewer cells viable to secrete MMP3 due to mortality from viral infection. It is possible that more MMP3 per infected cell was being secreted compared to uninfected cell.

MMP10 levels were also measured in supernatant from HFF-1 infected cells compared to uninfected cells (Figure 21). At 24 hours post infection, MMP10 was 2.3-fold higher in HCMV infected cells. MMP10 was elevated at 48 hours as well, and remained at the same level at 72 hours post infection, while uninfected cell MMP10 levels continued to increase, so that by 72 hours MMP10 levels were secreted less by HCMV infected cells than uninfected cells. These results demonstrate that HCMV infection increases release of MMP10 from HFF-1 cells immediately after infection. Considering that MMPs liberate growth factors and other MMPs from the extracellular matrix, this increase in MMP10 due to viral infection may have grave implications if the secretome from HCMV infected cells signal to breast cancer cells. Finally, no MMP2, MMP7 or MMP 9 was detected in supernatant from HFF-1 cells by ELISA. These experiments allow a better characterization of the changes to the secretome due to HCMV infection. These changes may directly impact tumor progression when an infected cell is nearby a breast cancer cell. To explore this impact, we collected supernatant from HFF-1 cells infected with HCMV and treated MCF-7 breast cancer cells with the conditioned medium, to determine changes in protein levels secreted from the tumor cells.

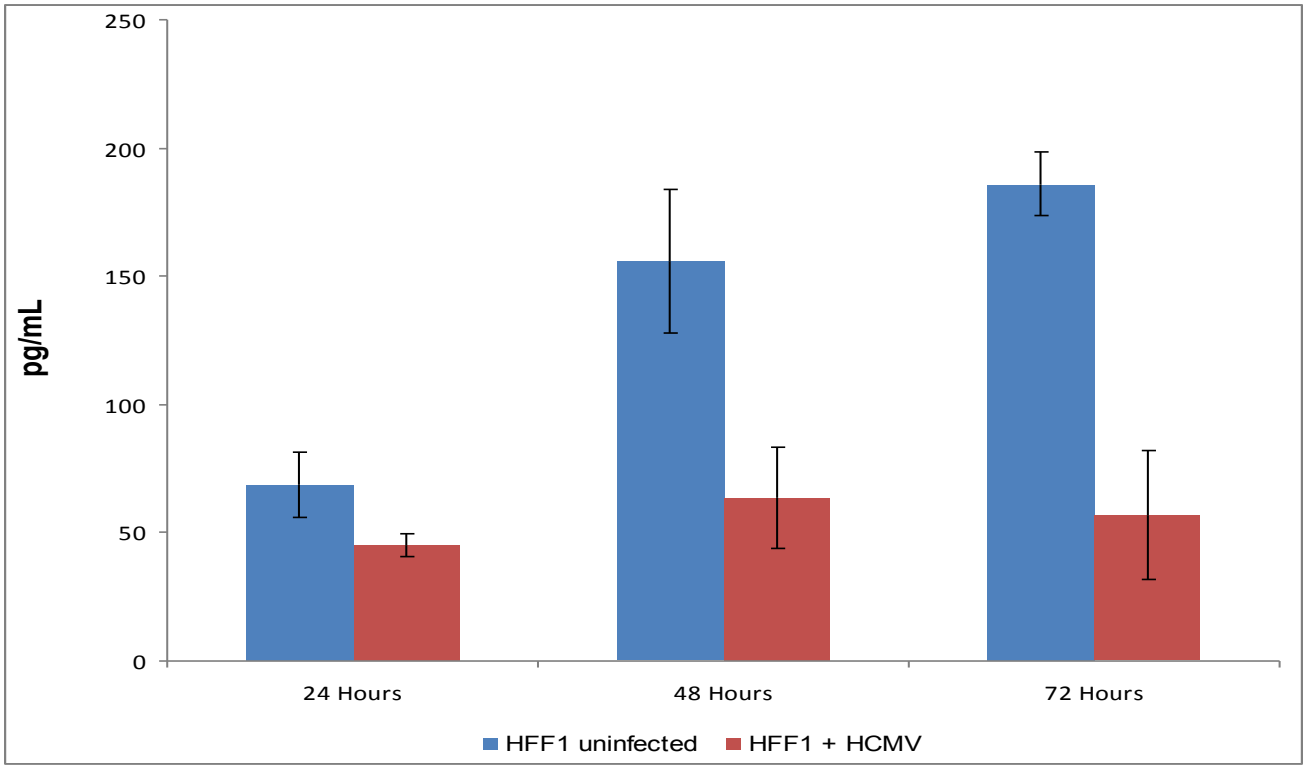

Figure 20. MMP3 concentration secreted from HFF-1 cells infected with HCMV.

Supernatant from HFF-1 cells infected with HCMV were analyzed for MMP3 protein levels by ELISA compared to supernatant uninfected HFF-1 cells. Error bars represent the range of concentrations measured for each condition. N = 3.

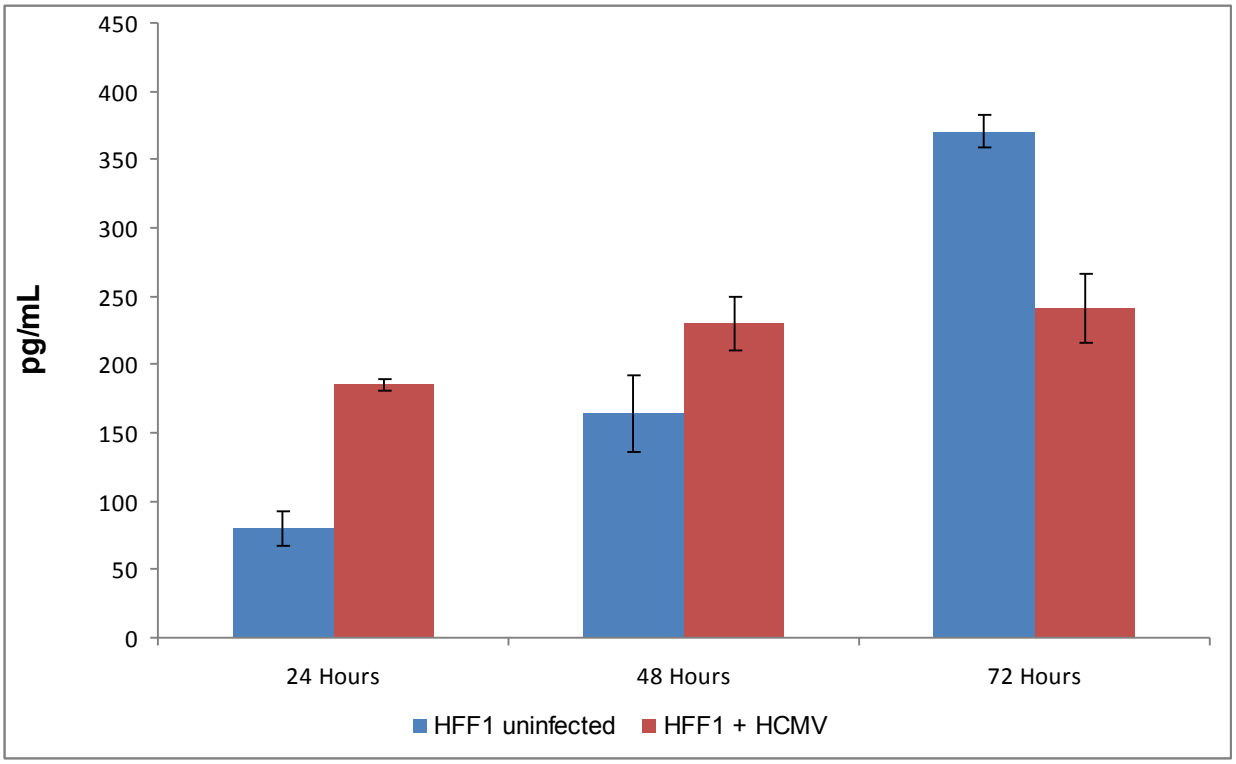

Figure 21. MMP10 concentration is increased after 24 and 48 hours post HCMV infection. Supernatant from HFF-1 cells infected with HCMV were analyzed for MMP10 protein levels by ELISA compared to supernatant uninfected HFF-1 cells. Error bars represent the range of concentrations measured for each condition. N = 3.

VI. Examination of the Secretome of MCF-7 Cells Treated with Conditioned Medium from HFF-1 Cells Infected with HCMV

Heterotypic signaling is the communication between dissimilar cell types. HCMV may alter the signaling molecules broadcasted by cells during communication, for example the increase in MMP10 levels secreted by HFF-1 cells due to HCMV infection. Considering the elevated levels of MMP transcripts in MCF-7 breast cancer cells due to treatment with cmvIL-10, yet no detectable MMP proteins in MCF-7 supernatant, we considered that the lack of secretion of MMPs may be due to the absence of a signaling factor from another cell. To investigate if MCF-7 cells would secrete more MMPs or other proteins related to cancer metastasis after receiving signals from HCMV infected cells, we treated MCF-7 cells with conditioned medium from HFF-1 cells infected with HCMV strain AD-169 and examined the secretome. An ELISA measuring MMP3 did not detect any MMP3 protein in the MCF-7 secretome after treatment with conditioned medium. Future experiments measuring for other MMPs may reveal that conditioned medium from HCMV infected cells unmasks factors in the tumor cell microenvironment, permitting MMPs to be secreted and detectable, and further indicating HCMV as a promoter of cancer progression.

We found that MCF-7 human breast cancer cells express the cellular IL-10 receptor, and treatment with cmvIL-10 alters expression of cell adhesion molecules and increases MMP gene expression. CmvIL-10 alters cellular activity by promoting cell migration. While MMP protein levels were not detected in the secretome of MCF-7 cells after treatment with cmvIL-10, HCMV infection increases MMP10 protein level secretion by HFF-1 cells, which may stimulate nearby breast cancer cells to metastasize more readily.

Discussion

The ability of cancer cells to invade other tissues and spread to organs throughout the body is detrimental to patient recovery. Between one-half and two-thirds of American women diagnosed with Stage II and III breast cancer will develop at least one secondary tumor within 5 years of diagnosis (39). Breast cancer tumors spread most frequently to the lymph nodes, lungs, liver and bones (50). Current research implicates HCMV as a contributor to some types of cancer progression (12, 43). Neuroblastoma cells infected with HCMV demonstrate down-regulation of adhesion molecules and exhibit increased motility (3). Additionally, HCMV has been detected in normal and neoplastic breast epithelium (23). The consequences of HCMV infection on the progression of breast cancer, however, have not been fully established.

To elucidate this connection, it was vital to examine the various mechanisms HCMV uses to manipulate the host cell environment (57). HCMV actively modulates multiple cell regulatory and signaling pathways in healthy, HCMV-seropositive humans (9). Manipulation of the host cell is achieved, in part, by the viral protein cmvIL-10. CmvIL-10, a powerful viral cytokine secreted from infected cells, is a homolog of human IL-10 with greater affinity than the human protein for the cellular IL-10 receptor (59). Research shows that cmvIL-10 effects are largely mediated through engagement of the IL-10R and subsequent activation of the Stat 3 pathway, which is primarily immunosuppressive (19, 64). These cellular changes could have vast implications in instances where cmvIL-10 is secreted near a breast tumor, as Stat 3 is associated with poor prognosis in ovarian cancer and considered a key factor in metastasis formation (79). Additionally, elevated levels of MMPs have been associated with poor prognoses in breast cancer progression (21). This thesis project examined cmvIL-10 and its effect on two factors

strongly associated with metastatic development, MMP activity and migration, in breast cancer progression.

We demonstrated that MCF-7 breast cancer cells display the human IL-10 receptor. Flow cytometry revealed cells express both Human IL-10Rα and IL-10Rβ on the cell surface (Figure 9). Additionally, immunofluorescence with permeablilization allowed visualization of human IL-10 receptor in a discrete punctate distribution throughout the cell (Figure 10). These results illustrated that human IL-10 receptor is available for binding on MCF-7 breast cancer cells and that, consequently, these cells could be sensitive to cmvIL-10 if present in the tumor microenvironment.

When these cells were treated with cmvIL-10, transcriptional expression of genes related to tumor metastasis was altered (Figure 11). Five genes related to metastasis were up-regulated more than 2-fold after both 24 and 48 hours of treatment with cmvIL-10, including FAT1, ITGB3, MCAM, SET, and SMAD2. The FAT1 and ITGB3 gene products are both involved in cell communication and migration (4, 52). MCAM is a multifunctional molecule called melanoma cell adhesion molecule, indicated in cell migration, angiogenesis and immune response (74). The SET gene product inhibits acetylation of nucleosomes to inhibit apoptosis (36). The SMAD2 gene product mediates TGF-β and acts as a transcription repressor (44). CmvIL-10 treatment consistently alters gene transcription of breast cancer cells over two days by distinctly up-regulating genes correlated to tumor progression and metastasis.

Additionally, treatment of breast cancer cells with cmvIL-10 led to modulations of adhesion-associated gene expression that involved a shift toward a transcript profile favoring more metastasis. Cell adhesion is important for tissue integrity and stability, and cancer cells that express less genes that permit cell adhesion may be more likely to detach from the primary

tumor. Cell adhesion genes FXYD5 and MCAM expression were up-regulated by treatment with cmvIL-10 (Figure 12). High levels of MCAM in human breast cancer cells are associated with loss of cell-cell contacts, expression of markers related to a phenotypic epithelial to mesenchymal transition, and increased cell motility (27, 76). FXYD, or domain containing ion transport regulator 5, also known as dysadherin, is a gene involved in the reduction of cell adhesion via E-cadherin as well as a biomarker for invasion (2). Also, APC, CDH1 and SYK cell-adhesion genes were down-regulated by treatment with cmvIL-10 (Figure 12). APC, or adenomatous polyposis cell, is a tumor suppressor gene indicated in inhibiting tumor invasiveness (53). SYK, or spleen tyrosine kinase, is a tumor suppressor gene that inhibits tumor growth, invasion and metastasis in breast cancer cells (67). Finally, the loss of function of CDH1, also known as cadherin 1 and E-cadherin, may contribute to tumor progression by increasing proliferation, invasion and metastasis (38). Therefore, the modulation of these cell adhesion genes demonstrates a shift in gene expression toward less adhesion-related proteins and an increased potential for movement away from the primary tumor. In this way, cmvIL-10 induces breast cancer cells to transcribe genes that enable detachment from the primary tumor, freeing them to begin the invasion process, as well as up-regulating many genes associated with migration and metastasis. Continuing research to determine if these gene expression changes translate into altered proteins levels may further elucidate the oncomodulatory activity of cmvIL-10.

Matrix metalloproteinases are proteolytic enzymes that degrade the extracellular matrix components collagen, fibronectin and laminin (73). Elevated levels of MMP-13 have been found to correlate with poor prognoses in breast cancer patients (77). In addition, evidence implicates the role of MMP-2 and MMP-9 in breast cancer genesis and progression (21, 66). Breast cancer

cells in the presence of cmvIL-10 demonstrated an up-regulation of genes encoding for MMPs (Figure 13), including MMP2, MMP3, MMP7, MMP9, MMP10, MMP11 and MMP13. An increase in degradation of extracellular components due to the presence of cmvIL-10 could provide malignant tumor cells access to the vascular and lymphatic systems, leading to further dissemination. The increase of MMP gene transcripts, combined with the modulation of cell adhesion and tumor progression genes, indicate cmvIL-10 promotes metastasis-related gene expression. CmvIL-10 alters gene transcription to allow cancer cells to break free of the primary tumor, migrate through the extracellular matrix and metastasize in the body.

Changes in gene transcription often correlate with altered protein levels in cells. To explore the changes in protein levels in breast cancer cells due to treatment with cmvIL-10, the secretome of MCF-7 cells was investigated. Considering the modification of metastasis-related gene expression observed, it was expected that cmvIL-10 treatment would cause changes to protein levels that would parallel the gene expression data, leading to an increase in MMPs secreted by the breast cancer cells. However, no MMP proteins were detected in cell supernatants; ELISAs did not detect MMP2, MMP3, MMP7, MMP9 or MMP10 in the supernatant from MCF-7 breast cancer cells. In interpreting this discrepancy, we considered that the MMP proteins might be being synthesized, but that they may be being inhibited by a TIMP. The cell supernatant was investigated for TIMP1, an inhibitor of MMPs, and plentiful TIMP1 was found to be secreted from MCF-7 cells (Figure 14). Treatment with cmvIL-10 did not, however, change the levels of TIMP1. It is possible that TIMP1, being abundant in the secretome, may be interfering with detection of the MMPs by ELISA. Another possibility is that while MMP protein levels are elevated in breast cancer cells in the presence of cmvIL-10, the proteins are not being secreted by the cells. MMPs are often cell associated, and membrane

type-MMPs (MT-MMPs) are implicated in breast cancer progression (34, 54). Future work could investigate MT-MMP protein levels in MCF-7 cell lysates to discern the impact of cmvIL-10 on MT-MMP protein levels in breast cancer cells.

Supernatants from breast cancer cells in the presence of cmvIL-10 were also explored for other factors related to metastasis. Our work shows that cmvIL-10 decreases levels of TGF-β in cell supernatants (Figure 15). While often considered a mitogenic protein, TGF-β is extremely versatile, contributing paradoxically to many cellular functions. One interesting mechanism of TGF-β is its connection with apoptosis. TGF-β is documented to induce apoptosis through the SMAD protein (29). Our work illustrates how cmvIL-10 can increase SMAD2 gene expression and decrease TGF-β protein levels, which may decrease the signaling for apoptosis, resulting in increased cancer cell viability. Future work to quantify SMAD2 protein levels in cell supernatant and deducing the cooperation of this protein with the reduced level of TGF- β proteins, might demonstrate cmvIL-10 reduces programmed cell death that could ultimately contribute to cancer progression.

Migration is a quantifiable cellular activity. Migration assays require that breast cancer cells migrate through a filter from one chamber to another, where they are harvested and counted. We showed that cmvIL-10 promotes MCF-7 breast cancer cell migration (Figure 18). Specifically, treatment with cmvIL-10 caused MCF-7 breast cancer cells to exhibit 138% more motility than cells not treated with the viral cytokine. The increased migration may be a result of elevated gene expression of migration-promoting genes such as those increased by cmvIL-10, FAT1, ITGB3, MCAM, and MMPs. These results showing modified cellular activity demonstrate that cmvIL-10, if secreted in the tumor microenvironment by infected cells, may increase breast cancer cell survival and invasion, resulting in an increased metastatic risk for cancer patients.

The modulation of gene expression and the robust change in cellular activity due to treatment with cmvIL-10 both implicate cmvIL-10 as a tumor-promoting molecule. One possibility for the lack of detection of secreted MMP proteins secreted from breast cancer cells, despite a robust up-regulation in MMP gene expression, could be due to the absence of a signaling factor from another cell. Heterotypic signaling is the communication between dissimilar cell types. HCMV may alter the signaling molecules broadcasted by cells during communication. HCMV infected cells may secrete multiple factors that work conjointly, which may be necessary to signal MCF-7 cells to secrete MMPs. To investigate if MCF-7 cells would secrete more MMPs or other proteins related to cancer metastasis after receiving signals from HCMV infected cells, we first infected human foreskin fibroblasts with HCMV and characterized the supernatants from HCMV infected cells.

We found that HCMV actively modulates the secretome of HCMV infected HFF-1 cells. Fibroblasts infected with HCMV secreted more MMP10 protein than uninfected HFF-1 cells (Figure 21). An increase in MMP10 in the tumor microenvironment might contribute to the degradation of the extracellular matrix surrounding the cancer cells, promoting tumor invasion. Additionally, this increase in MMP10 protein level could further transform the tumor microenvironment by unmasking other factors in the surrounding extracellular matrix that enhance tumor invasion, such as the metastasis-promoting proteins VEGF or TGF-β. As MMPs degrade the extracellular matrix, metastasis-related proteins that are suspended in the meshwork in the matrix become liberated and will signal breast cancer cells to proliferate, detach from the primary tumor and invade other tissues (22). In addition to the degradation and remodeling of the ECM, MMPs unveil adhesion molecule sites that are usually embedded within the matrix, which become available for cell attachment, permitting migration (55). Furthermore, the altered

secretome from HCMV infected cells and the unmasked factors in the tumor microenvironment may cooperate with cmvIL-10. It is possible that a mélange of virally-encoded proteins, including cmvIL-10, and the increased availability of secreted cellular proteins such as MMP10 from HCMV infected cells, as well as the unmasked factors from the remodeled ECM, work conjunctively to coordinate a heterotypic signal that induces a metastatic phenotype in the breast cancer cell.

Likely, the effects of HCMV on cancer cell metastasis are additive. This research demonstrates cmvIL-10 plays a stimulatory role in cancer cell migration. CmvIL-10 also contributes to a gene expression profile that favors metastasis. While cmvIL-10 increases MMP gene expression in MCF-7 breast cancer cells, additional secreted factors from HCMV infected cells may be needed to induce secretion of the MMPs. Possibly, mRNA transcripts are never translated, perhaps due to RNA interference. Continuing research to further identify proteins in the secretome of HCMV infected cells could reveal additional factors that contribute to MMP secretion by breast cancer cells. Finally, future work treating MCF-7 cells with conditioned medium from HCMV infected cells may reveal the anticipated elevated MMP protein levels in the breast cancer cell secretome, as suggested by qRT-PCR results.

HCMV orchestrates extensive manipulations of cellular pathways that result in abundant micro-effects in the host organism. While the majority of available research illustrates cmvIL-10 as a suppressive regulatory cytokine, we show here it exhibits an amplification effect, specifically for genes encoding for MMPs and activities like cell migration. These results indicate cmvIL-10 plays an oncomodulatory role through the enhancement of breast tumor metastasis.

A full 15% of all cancer-related deaths in women are from breast cancer (39). This alarming statistic may be lowered through continued research and discovery. We show that cmvIL-10 contributes to cell invasion, thus supporting and expanding on previous studies that implicated HCMV as an oncomodulatory virus. The correlation of HCMV, STAT3, and high MMP levels with increased potential for tumor metastasis are supported by this study. Experiments to determine how a latent infection of HCMV affects MMP protein activity would also benefit this body of knowledge. Further research testing the effect of HCMV infection on metastasis in animal models will supplement this research project. In a clinical setting, antiviral therapy may be considered in conjunction with chemotherapy efforts for patients who present with highly metastatic tumors.

Appendix

Table 2. Human Tumor Metastasis qRT-PCR Gene Array. A list of the 84 metastasis-related genes analyzed via qRT-PCR using the RT^2 Profiler Human Tumor Metastasis Array.

Gene Symbol	Description	Gene Name
APC	Adenomatous polyposis coli	BTPS2, DP2, DP2.5, DP3, GS
BRMS1	Breast cancer metastasis suppressor 1	DKFZp564A063
CCL7	Chemokine (C-C motif) ligand 7	FIC, MARC, MCP-3, MCP3, MGC138463, MGC138465, NC28, SCYA6, SCYA7
CD44	CD44 molecule (Indian blood group)	CDW44, CSPG8, ECMR-III, HCELL, HUTCH-I, IN, LHR, MC56, MDU2, MDU3, MGC10468, MIC4, Pgp1
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Arc-1, CD324, CDHE, ECAD, LCAM, UVO
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	CAD11, CDHOB, OB, OSF-4
CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	CAD6, DKFZp564N1116, FLJ14176, KCAD
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	ARF, CDK4I, CDKN2, CMM2, INK4, INK4A, MLM, MTS-1, MTS1, P14, P14ARF, P16, P16-INK4A, P16INK4, P16INK4A, P19, P19ARF, TP16
CHD4	Chromodomain helicase DNA binding protein 4	DKFZp686E06161, Mi-2b, Mi2-BETA
COL4A2	Collagen, type IV, alpha 2	DKFZp686I14213, FLJ22259
CST7	Cystatin F (leukocystatin)	CMAP
CTBP1	C-terminal binding protein 1	BARS, MGC104684
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	CAP102, FLJ36832, FLJ52416
CTSK	Cathepsin K	CTS02, CTSO, CTSO1, CTSO2, MGC23107, PKND, PYCD
CTSL1	Cathepsin L1	CATL, CTSL, FLJ31037, MEP
CXCL12	Chemokine (C-X-C motif) ligand 12	IRH, PBSF, SCYB12, SDF1, SDF1A, SDF1B, TLSF, TPAR1
CXCR4	Chemokine (C-X-C motif) receptor 4	CD184, D2S201E, FB22, HM89, HSY3RR, LAP3, LCR1, LESTR, NPY3R, NPYR, NPYRL, NPYY3R, WHIM
DENR	Density-regulated protein	DRP, DRP1, SMAP-3
EPHB2	EPH receptor B2	CAPB, DRT, EK5, EPHT3, ERK, Hek5, MGC87492, PCBC, Tyro5
ETV4	Ets variant 4	E1A-F, E1AF, PEA3, PEAS3
EWSR1	Ewing sarcoma breakpoint region 1	EWS, bK984G1.4
FAT1	FAT tumor suppressor homolog 1 (Drosophila)	CDHF7, CDHR8, FAT, ME5, hFat1
FGFR4	Fibroblast growth factor receptor 4	CD334, JTK2, MGC20292, TKF
FLT4	Fms-related tyrosine kinase 4	FLT41, LMPH1A, PCL, VEGFR3
FN1	Fibronectin 1	CIG, DKFZp686F10164, DKFZp686H0342, DKFZp686I1370, DKFZp686O13149, ED-B, FINC, FN, FNZ, GFND, GFND2, LETS, MSF
FXYD5	FXYD domain containing ion transport regulator 5	DYSAD, IWU1, KCT1, OIT2, PRO6241, RIC
GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	GNRH, GRH, LHRH, LNRH
KISS1R	KISS1 receptor	AXOR12, GPR54, HOT7T175
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	DFNB39, F-TCF, HGFB, HPTA, SF
HPSE	Heparanase	HPA, HPA1, HPR1, HPSE1, HSE1
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	C-BAS, HAS, C-H-RAS, C-HA-RAS1, CTLO, H-RASIDX, HAMSV, HRAS1, K-RAS, N-RAS, RASH1
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	CC3, FLJ26963, SDR44U1, TIP30
IGF1	Insulin-like growth factor 1 (somatomedin C)	IGF-I, IGF1A, IGFI
IL18	Interleukin 18 (interferon-gamma-inducing factor)	IGIF, IL-18, IL-1g, IL1F4, MGC12320

IL1B	Interleukin 1, beta	IL-1, IL1-BETA, IL1F2
CXCR2	Chemokine (C-X-C motif) receptor 2	CD182, CDw128b, CMKAR2, IL8R2, IL8RA, IL8RB
ITGA7	Integrin, alpha 7	FLJ25220
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	CD61, GP3A, GPIIIa
CD82	CD82 molecule	4F9, C33, GR15, IA4, KAI1, R2, SAR2, ST6, TSPAN27
KISS1	KiSS-1 metastasis-suppressor	KiSS-1, METASTIN, MGC39258
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	C-K-RAS, K-RAS2A, K-RAS2B, K-RAS4A, K-RAS4B, KI-RAS, KRAS1, KRAS2, NS, NS3, RASK2
RPSA	Ribosomal protein SA	37LRP, 67LR, LAMBR, LAMR1, LBP, LBP, p40, LRP, LRP, LR, NEM, 1CHD4, SA, lamR, p40
MCAM	Melanoma cell adhesion molecule	CD146, MUC18
MDM2	Mdm2 p53 binding protein homolog (mouse)	HDMX, MGC5370, MGC71221, hdm2
MET	Met proto-oncogene (hepatocyte growth factor receptor)	AUTS9, HGFR, RCCP2, c-Met
METAP2	Methionyl aminopeptidase 2	MAP2, MNPEP, p67, p67eIF2
MGAT5	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	GNT-V, GNT-VA
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	SL-2, STMY2
MMP11	Matrix metallopeptidase 11 (stromelysin 3)	SL-3, ST3, STMY3
MMP13	Matrix metallopeptidase 13 (collagenase 3)	CLG3, MANDP1
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4, CLG4A, MMP-II, MONA, TBE-1
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	CHDS6, MGC126102, MGC126103, MGC126104, MMP-3, SL-1, STMY, STMY1, STR1
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	MMP-7, MPSL1, PUMP-1
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B, GELB, MANDP2, MMP-9
MTA1	Metastasis associated 1	-
MTSS1	Metastasis suppressor 1	DKFZp781P2223, FLJ44694, KIAA0429, MIM, MIMA, MIMB
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	MRTL, bHLHe39, c-Myc
MYCL1	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	LMYC, MYCL, bHLHe38
NF2	Neurofibromin 2 (merlin)	ACN, BANF, SCH
NME1	Non-metastatic cells 1, protein (NM23A) expressed in	AWD, GAAD, NB, NBS, NDKA, NDPK-A, NDPKA, NM23, NM23-H1
NME2	Non-metastatic cells 2, protein (NM23B) expressed in	MGC111212, MGC2212, NDKB, NDPK-B, NDPKB, NM23-H2, NM23B, PUF
NME4	Non-metastatic cells 4, protein expressed in	NDPK-D, NM23H4, nm23-H4
NR4A3	Nuclear receptor subfamily 4, group A, member 3	CHN, CSMF, MINOR, NOR1, TEC
PLAUR	Plasminogen activator, urokinase receptor	CD87, U-PAR, UPAR, URKR
PNN	Pinin, desmosome associated protein	DRS, DRSP, SDK3, memA
PTEN	Phosphatase and tensin homolog	10q23del, BZS, DEC, GLM2, MGC11227, MHAM, MMAC1, PTEN1, TEP1
RB1	Retinoblastoma 1	OSRC, RB, p105-Rb, pRb, pp110
RORB	RAR-related orphan receptor B	NR1F2, ROR-BETA, RZR-BETA, RZRB, bA133M9.1
SET	SET nuclear oncogene	2PP2A, I2PP2A, IGAAD, IPP2A2, PHAPII, TAF-I, TAF-IBETA
SMAD2	SMAD family member 2	JV18, JV18-1, MADH2, MADR2, MGC22139, MGC34440, hMAD-2, hSMAD2
SMAD4	SMAD family member 4	DPC4, JIP, MADH4
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	ASV, SRC1, c-SRC, p60-Src
SSTR2	Somatostatin receptor 2	-
SYK	Spleen tyrosine kinase	DKFZp313N1010, FLJ25043, FLJ37489

TCF20	Transcription factor 20 (AR1)	AR1, KIAA0292, SPBP
TGFB1	Transforming growth factor, beta 1	CED, DPD1, LAP, TGFB, TGFbeta
TIMP2	TIMP metallopeptidase inhibitor 2	CSC-21K
TIMP3	TIMP metallopeptidase inhibitor 3	HSMRK222, K222, K222TA2, SFD
TIMP4	TIMP metallopeptidase inhibitor 4	-
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	APO2L, Apo-2L, CD253, TL2, TRAIL
TP53	Tumor protein p53	FLJ92943, LFS1, P53, TRP53
TRPM1	Transient receptor potential cation channel, subfamily M, member 1	CSNB1C, LTRPC1, MLSN1
TSHR	Thyroid stimulating hormone receptor	CHNG1, LGR3, MGC75129, hTSHR-I
VEGFA	Vascular endothelial growth factor A	MGC70609, MVCD1, VEGF, VPF
B2M	Beta-2-microglobulin	-
HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
RPL13A	Ribosomal protein L13a	L13A, TSTA1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685
ACTB	Actin, beta	PS1TP5BP1
HGDC	Human Genomic DNA Contamination	HIGX1A
RTC	Reverse Transcription Control	RTC
RTC	Reverse Transcription Control	RTC
RTC	Reverse Transcription Control	RTC
PPC	Positive PCR Control	PPC
PPC	Positive PCR Control	PPC
PPC	Positive PCR Control	PPC

*SABiosciences website (http://www.sabiosciences.com/genetable.php?pcatn=PAHS-028A)

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